Effects of sulphite on yeasts with special reference to intracellular buffering capacity

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Submitted by Shane Patrick Maloney

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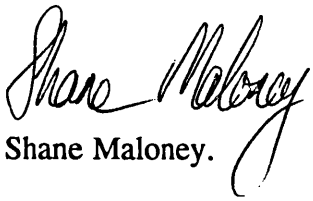

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SUMMARY

One strain of *Saccharomyces cerevisiae* and two strains of *Saccharomycodes ludwigii*, namely *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10, were chosen in order to study their reactions to sulphite. Both strains of *S'codes ludwigii* were found to be considerably more tolerant of sulphite than *Sacch. cerevisiae* AWRI 1A65; this was quantified using microtitre-plate analysis and also by measurement of growth in one-litre portions of sulphite-supplemented media. Sub-lethal concentrations of sulphite were defined for each yeast strain. Production of the sulphite-binding compound, acetaldehyde, in response to sub-lethal concentrations of sulphite was studied. *Saccharomyces cerevisiae* AWRI 1A65 did not produce sufficient acetaldehyde to bind all free sulphite present. Both strains of *S'codes ludwigii* produced acetaldehyde in concentrations which were equimolar to concentrations of free sulphite present in the medium.

Experiments investigating aspects of buffering capacity were conducted, and included a study of yeast-cell responses to acidification. Unbuffered suspensions of *Sacch. cerevisiae* AWRI 1A65 demonstrated declines in pH value in response to acidification; suspensions of either strain of *S'codes ludwigii* resisted declines in pH value. Responses of *S'codes ludwigii* to acidification could not be correlated to any particular fraction of yeast cells. Studies of intracellular buffering capacities revealed that *Sacch. cerevisiae* AWRI 1A65 possessed a greater resistance to acidification than either strain of *S'codes ludwigii*. Two methods for measuring intracellular buffering capacity were used. One method involved titration of isolated cell cytosols against acid, and the other utilized data from uptake-experiments with radioactively-labelled sulphite or propionic acid.

Saccharomyces cerevisiae AWRI 1A65 was found to accumulate much greater concentrations of sulphite than either of the *S'codes ludwigii* strains. In addition, *Sacch. cerevisiae* AWRI 1A65 was found to have a greater intracellular pH value than either *S'codes ludwigii* strain, as measured using radioactively-

labelled propionic acid. Cells harvested from different phases of growth were found to possess different intracellular pH values and buffering capacities. Generally, relatively low intracellular pH values and small intracellular buffering capacities were associated with the stationary phase of growth.

Sulphite resistance in yeasts was associated with three factors: (i) small accumulation of sulphite, due to (ii) relatively low intracellular pH values and (iii) relatively small intracellular buffering capacities.

CONTENTS.

CONTENTS

ACKNOWLEDGEMENTS.

I would like to take this opportunity to express my gratitude to my supervisor, Professor A.H. Rose, for his help and guidance throughout the trials and tribulations of this work. I would like to thank my sponsors, HP Bulmer, for their willingness to assist in studies of cider-related matters. In the Zymology laboratory, in addition to providing considerable technical expertise, Felicity Veazey demonstrated a patience I had never previously witnessed and so deserves special thanks. Those who have contributed to the upkeep of my sanity and kept me going with support (financial and otherwise), kindness and humour, you know who you are, thanks.

INTRODUCTION.

INTRODUCTION

Before the main body of Introduction embellishes it, the rationale behind this thesis can be expressed as follows. *Saccharomycodes ludwigii* is extremely resistant to the preservative sulphite, significantly more so than *Saccharomyces cerevisiae*. In cider-making, where sulphite is the primary form of yeast control, it is the presence of *Sacch. cerevisiae* and not *S'codes ludwigii* which is desired during fermentation. In addition, sulphite is used to protect packaged cider from microbial spoilage, and the main contender to cause such spoilage is *S'codes ludwigii*. For these reasons, sulphite, cider-making, *Sacch. cerevisiae*, *S'codes ludwigii*, and the links between these form the subject matter for this thesis. In this Introduction, fundamental matters of sulphite chemistry and reactivity will be dealt with first, followed by a brief survey of general uses for the preservative. An account of cider manufacture, the *raison d'etre* for this project, is given before the central issue of sulphite resistance in yeast is addressed.

PROPERTIES OF SULPHUR DIOXIDE AND SULPHITES.

Sulphur reacts quite readily with oxygen or air to form a number of oxides of sulphur. However, when sulphur is burned in air, the main product is sulphur dioxide (SO_2) along with small amounts of sulphur trioxide (SO_3). Combustion of elemental sulphur constitutes the major form of industrial manufacture of sulphur dioxide. It is produced in greater quantities than any other single sulphur compound, and 98 % of this production is channelled into the manufacture of sulphuric acid (Sander *et al.*, 1984). The commercial and industrial importance of sulphur dioxide has led to extensive investigations of its properties. It is a colourless but irritating gas, and is easily detectable by the human nose because of its pungent, suffocating smell. The sulphur dioxide molecule, due to resonance,

probably has two structures: $\bar{O}\text{-}\overset{+}{S}\text{=}O$ and $O\text{=}\overset{+}{S}\text{-}\bar{O}$. The sulphur-oxygen distance is 0.143 nm, and the bond angle O-S-O is 119.5°. Sulphur dioxide melts at -75.5°C and boils at -10°C. Gaseous sulphur dioxide dissolves readily in water to form, in theory, sulphurous acid H_2SO_3. The solution possesses well-known acidic properties, conducts electricity and behaves as if it contains an acid. However, there is no evidence that the free acid exists in solution. Ultraviolet absorption spectrum studies indicate that, in aqueous solution, sulphurous acid consists largely of uncombined sulphur dioxide molecules (Mellor, 1930). Infrared and Raman spectroscopy confirm that sulphur dioxide molecules are not strongly hydrated in aqueous solution and do not form the compound H_2SO_3 (Falk and Giguere, 1958). Instead, dissolved sulphur dioxide forms a clathrate in which the SO_2 molecule is enclosed inside a water shell, without chemical bond formation (Gould and Russell, 1991). The structure is usually represented as $SO_2 \cdot x\ H_2O$. In solution, molecular sulphur dioxide dissociates to form the bisulphite anion HSO_3^-:

$$SO_2 \cdot H_2O \rightleftharpoons HSO_3^- + H^+$$

Bisulphite can dissociate further to give the sulphite anion SO_3^{2-}:

$$HSO_3^- \rightleftharpoons SO_3^{2-} + H^+$$

The first dissociation constant, leading to ionization of sulphur dioxide, is well established at 1.70×10^{-2} (pKa = 1.77; King *et al.*, 1981). This value is dependent on conditions, and slightly different values (pKa = 1.76, 1.79, 1.86, 1.90) have been published (Mellor, 1930; Rose and Pilkington, 1989; Gould and Russell, 1991). The second dissociation constant for the reaction leading to production of the sulphite anion, under the same conditions, is 6.31×10^{-8} (pKa = 7.20; King *et al.*, 1981). A value for the second dissociation constant of pKa = 5.30, published by Vas and Ingram (1949), has become disseminated in the literature and leads to erroneous results if used to calculate proportions of sulphur dioxide, bisulphite and sulphite present in solution. However, with pKa values of 1.77 and 7.20 for the

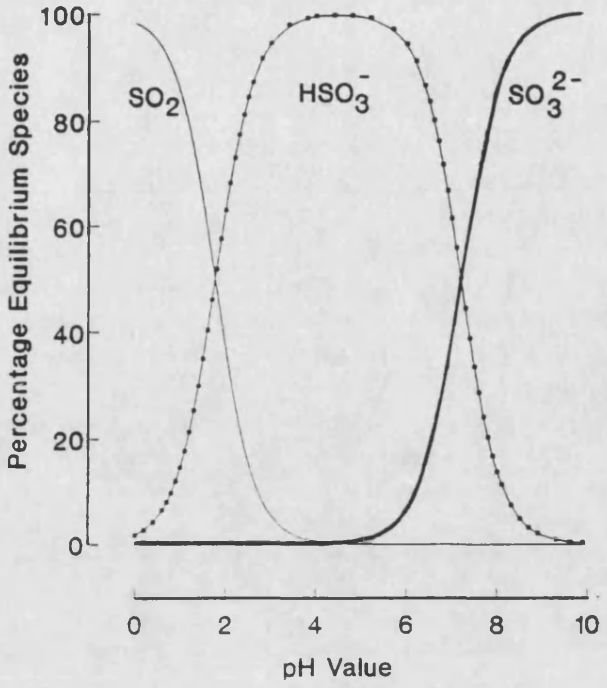


Figure 1. Percentage distribution of equilibrium species of sulphur dioxide, in aqueous solution, as a function of pH value.

first and second dissociations, respectively, the correct proportions of each molecular species can be calculated as a function of pH value (Appendix 1). A plot of these data is given in Figure 1. The presence of molecular sulphur dioxide is favoured at low pH values, the sulphite anion at high pH values, and the bisulphite anion at intermediate values.

The dissociations described above occur in dilute solutions, as is the case with apple juice and cider. At higher concentrations, the bisulphite anion can condense to form the metabisulphite (disulphite) anion $S_2O_5^{2-}$:

$$2\ HSO_3^- \rightleftharpoons S_2O_5^{2-} + H_2O$$

A variety of sulphur salts can be used to generate aqueous sulphur dioxide, and those most commonly employed are sodium or potassium metabisulphites. A metabisulphite anion, in dilute solution, dissociates to form the bisulphite anion. This in turn is in pH-dependent equilibrium with sulphite and sulphur dioxide, summarized as follows:

$$S_2O_5^{2-} \rightleftharpoons HSO_3^- \rightleftharpoons SO_3^{2-} \rightleftharpoons SO_2 \cdot H_2O$$

Compounds such as sodium or potassium metabisulphite are safer, and more convenient to handle, than gaseous or liquid sulphur dioxide and have, along with sulphite and bisulphite salts, superseded anhydrous sulphur dioxide as the main means of generating aqueous sulphite.

REACTIONS OF SULPHITE.

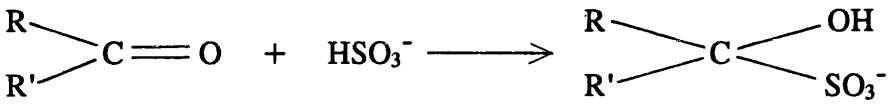
Sulphur dioxide and its related anions are very reactive molecules, able to both donate and accept electrons. It would be possible to document a very wide range of reactions which could be carried out *in vitro*, but this would not be meaningful in the context of this Introduction. Only those reactions which are of biological significance will be considered here.

(a) Oxygen. The sulphite anion is oxidized by molecular oxygen to sulphate, as follows:

$$2\ SO_3^{2-} + O_2 \rightarrow 2\ SO_4^{2-}$$

The bisulphite anion is less easily oxidized by oxygen, and molecular sulphur dioxide reacts only to a very small extent if at all. Subsequently, by lowering the pH value of a solution, conversion of sulphite to sulphate can be minimized. During oxidation of sulphite by oxygen, it is thought that the free radicals $SO_3^{-}\bullet$ and $\bullet O_2$ may be formed as intermediates. Micro-organisms possess enzymes, such as superoxide dismutase, which mop up free radicals. However, it is possible that the killing effects which sulphite exhibits to a greater or lesser extent in micro-organisms could be due, in part, to highly reactive free radicals.

(b) Carbonyl Groups. Perhaps the best-known reaction of sulphite is the addition of HSO_3^- to carbonyl groups. The carbonyl groups of aldehydes and ketones are found in many biologically important molecules including proteins and some enzyme cofactors. Sulphite reacts with open-chain carbonyl compounds to form hydroxysulphonate compounds (Hammond and Carr, 1976), as follows:



All aldehydes form hydroxysulphonates, but not all ketones. Diethyl ketone reacts slowly and to a limited extent. Otherwise, only ketones with a methyl group adjacent to the carbonyl or carbonyls which are part of a four- to seven- member carbon ring will react. Combination of sulphite with sugars is limited to those with a free aldehyde group. Such reactions are slower than with aldehydes and ketones, and the products formed are relatively more unstable. Sulphite reacts most readily with galactose, mannose and arabinose; less readily with maltose, lactose and glucose; very slowly with raffinose, and does not react at all with sucrose or fructose (Joslyn and Braverman, 1954). Equilibrium constants for the dissociation of certain carbonyl compounds are given in Table 1. The equilibrium constants express the ratio of the velocity constants of the dissociation and formation reactions of the reversible carbonyl-bisulphite system. The equilibrium constants of these complexes lie between those of acetaldehyde and glucose. Burroughs and Sparks (1973) found that values of K remain essentially constant between pH 3 and 6, which means that the velocity constants for both dissociation and formation reactions are equally affected over this pH range, a range sufficient to account for most foods and beverages. Burroughs and Whiting (1960) determined the sulphur dioxide-binding powers of certain carbonyl compounds in terms of percentage of total compound present which is bound; they found that acetaldehyde combines most avidly with sulphite (Table 1). In ciders, acetaldehyde is quantitatively the most important sulphur dioxide-binding constituent (Burroughs and Whiting, 1960).

Carbonyl Compound	Equilibrium Constant (K)	% SO_2-Binding Power
Acetaldehyde	1.4×10^{-6}	100
Pyruvic acid	2.2×10^{-4}	66
α-Ketoglutaric acid	7.0×10^{-4}	47
L-Xylosone	1.4×10^{-3}	27
Galactouronic acid	2.1×10^{-2}	2.5
Glucose	6.4×10^{-1}	0.12

Table 1. Apparent equilibrium constants at pH 4.0, and SO_2-binding power (bound carbonyl compound as a percentage of total present, at 50 p.p.m. free sulphite) of carbonyl-bisulphite compounds. Values compiled from the data of Burroughs and Whiting (1960) and Burroughs and Sparks (1973).

(c) **Proteins.** Reaction of sulphite with carbonyl compounds, which may be present in proteins, was discussed in the previous section. Virtually all proteins contain disulphide links between cysteine residues which help to stabilize polypeptide tertiary structure. Sulphite reacts with disulphide bonds in free cysteine to produce a cysteine-thiosulphonate (Ough, 1983):

$$R_1 - S - S - R_2 \quad + \quad SO_3^{2-} \longrightarrow R_1 - S - SO_3^- \quad + \quad R_2S^-$$

Under non-denaturing conditions and at physiological pH values, the reaction between sulphite and free cysteine goes to completion, while the disulphide bonds of most proteins are unreactive (Gunnison, 1981). The unreactive protein disulphide bonds are probably protected from nucleophilic attack either through steric hindrance, or by unfavourable electronic environments of neighbouring amino

acids. However, it remains a possibility that an enzyme could be inactivated by a change in conformation of protein tertiary structure, caused by reaction of disulphide links with sulphite.

(d) Coenzymes and Prosthetic Groups. Inhibition of enzyme activity may be attributable on occasions to inactivation of a coenzyme which is essential for enzyme activity. Two coenzymic forms of niacin are nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). These molecules have within their structure a pyridine residue, with which sulphite reacts. The reaction can be summarized as follows:

$$NAD^+ \quad + \quad SO_3^{2-} \longrightarrow NAD\ SO_3^-$$

The adduct formed with sulphite is inactive. The stability of the adduct is only moderate ($K = 3 \times 10^{-2}$); however, when NAD^+ is associated with certain enzymes, the sulphite adduct becomes more stable (Gunnison, 1981). Micro-organisms rely heavily on $NADP^+$-dependent dehydrogenases, and it follows that sulphite will disrupt their normal function (Gould and Russell, 1991). Tightly bound coenzymes are referred to as prosthetic groups. Two prosthetic groups concerned in oxidation-reduction reactions, namely flavins and haems, are both inactivated by sulphite. Other susceptible prosthetic groups are the vitamins folic acid, pyridoxal and thiamin. Folic acid is thought to be inactivated by the bisulphite anion. Thiamin is cleaved irreversibly by bisulphite into its pyrimidine and thiazole residues. It is thought that the carbonyl group of pyridoxal is involved in reaction with sulphite (Rose and Pilkington, 1989).

(e) Nucleic Acids. The pyrimidine bases cytosine, thymine and uracil are subject to attack by sulphite. For example, cytosine is transformed to cytidine by the bisulphite anion (Ough, 1983). In addition, the purine adenine, which is present in DNA and RNA as well as in adenine dinucleotides (ATP, ADP) and cofactors

(NAD^+, FAD), forms addition compounds with sulphite. In theory, these reactions would terminate the polymerization of nascent DNA on a DNA template, as well as the transcription of DNA into RNA, and inhibit the process of translation. Such reactions can be demonstrated *in vitro*, but their significance *in vivo* is speculative.

APPLICATIONS OF SULPHITE IN FOODS AND BEVERAGES.

Sulphur dioxide and sulphite salts are listed as preservatives, designated E 220 - 227, in directives of the European Economic Community (Hanssen and Marsden, 1991). The major categories of sulphite use are listed in Table 2. Sulphite plays a crucial role in the inhibition of yeasts, bacteria and moulds in many foods and beverages. Sulphite is used to prevent post-harvest deterioration of fruits used for juice production. It is employed to minimize the incidence of infections of *Botrytis* species on soft fruits, such as strawberries and raspberries, prior to jam production (Roberts and McWeeny, 1972). In meat products, sulphite is effective in delaying growth of moulds and Gram-negative bacteria; inhibition of the last group, which includes salmonellae, is of particular importance as such organisms compromise consumer safety (Gould and Russell, 1991). Sulphite is often added to fruit juices before fermentation to inhibit the growth of bacteria and wild yeasts, allowing subsequent controlled fermentations to be initiated with pure cultures of a chosen yeast strain. The sensitivity of individual yeast species varies widely; *Pichia membranaefaciens* and *Kloeckera apiculata* are regarded as sulphite-sensitive, whereas *Zygosaccharomyces bailii*, *Brettanomyces* species and *S'codes ludwigii* are regarded as resistant (Hammond and Carr, 1976). *Saccharomycodes ludwigii* is most notable as a spoilage yeast of apple juices and ciders which have been treated with sulphite. Its role as a spoilage yeast and its interaction with sulphite will be discussed in detail later in this Introduction. As an anti-oxidant, sulphite is particularly effective when used to prevent loss of ascorbic acid during processing

Role	Function
Antimicrobial agent	Control of fruit juice fermentations by inhibition of non-fermenting yeasts and bacteria. Inhibition of non-pathogenic and pathogenic organisms in meat products. Post-harvest preservation of soft fruits.
Anti-oxidant	Prevents loss of oxidizable compounds during storage. Minimizes organoleptic and colour changes of products.
Inhibitor of enzymic and non-enzymic reactions	Prevents enzymic and non-enzymic formation of dark-coloured pigments.
Bleaching agent	Reacts with coloured compounds to give lighter-coloured products.

Table 2. Major categories of use of sulphite.

and storage of such widely differing products as dehydrated cabbage, fruit cordials, grape juice and tomato puree (Taylor *et al.*, 1986). Sulphite prevents oxidation of essential oils and carotenoids in citrus juices, and in doing so prevents development of off-flavours and loss of colour. Beverages, such as beers and ciders, are

protected by sulphite from oxidative changes in flavour due to dissolved oxygen (Roberts and McWeeny, 1972).

Non-enzymic browning is a term used to describe a family of reactions that commonly involve the formation of carbonyl intermediates and brown, polymeric pigments; the flavour of the product may also be altered (Wedzicha, 1991). Examples of non-enzymic browning reactions involve the combination of amino acids and reducing sugars, and caramelization of sugars. Sulphite reacts with the carbonyl intermediates and thus blocks formation of brown pigments (Taylor *et al.*, 1986).

An example of enzymic browning is the oxidation of diphenols to quinones by the enzyme polyphenoloxidase. Quinones can cyclize, undergo further oxidation and condense to form brown pigments. The mechanism by which sulphite prevents pigment formation is not clear. It is possible that it inhibits the enzyme directly; or combines with quinones to prevent cyclization, or it may act as a reducing agent to convert quinones back to diphenols.

The reducing properties of sulphite are important in its bleaching action; sulphite reacts with some coloured compounds such that they are rendered colourless (Mellor, 1930).

SAFETY OF SULPHITES IN FOODS AND BEVERAGES.

Adverse reactions to sulphite in non-asthmatic individuals appear to be extremely rare (Bush *et al.*, 1986). However, evidence that sulphites can provoke bronchospasms in asthmatics is convincing. Case studies of sulphite-induced, life-threatening asthmatic attacks have been published (Baker *et al.*, 1981), but it should be stressed that such serious reactions only occur in acutely asthmatic individuals. Unfortunately, it is inevitable that, in the modern health-concious society, there will be concern about the safety of sulphites in foods and beverages (Millstone, 1984).

Ill effects are mainly related to irritation and damage of moist mucous membranes, by formation of acidic solutions. Inhalation studies indicate that doses of 6 - 12 mg l^{-1} cause immediate irritation of the respiratory tract in normal individuals, and bronchospasms in asthmatic individuals (Bush *et al.*, 1986). Any sulphite absorbed by the body is converted to sulphate by the enzyme sulphite oxidase. Most mammalian tissues contain sulphite oxidase, which functions to oxidize sulphite produced endogenously by the breakdown of sulphur-containing amino acids. Sulphate is rapidly excreted in the urine, as evidenced by an increase in urine acidity.

It is difficult to estimate accurately the intake of sulphite in the human diet. The concentrations used to treat foods and beverages initially do not reflect the amounts present after processing and storage. In addition, there are wide variations in individual food-consumption patterns (Anonymous, 1975). Green (1976) concluded that sulphite is no more undesirable an additive than a naturally occurring substance, and its use would only be dangerous if high concentrations were consumed; the deleterious effects which sulphite would have upon the flavour and colour of foods and beverages, if used in disproportionate amounts, provide a safeguard against indiscriminate use.

MANUFACTURE OF CIDER.

A cider is a beverage produced by partial or complete fermentation of apple juice. In legal terms, to be considered a cider, the alcoholic strength must lie between 1.2 and 8.5 % (v/v) at 20°C (National Association of Cider Makers, 1988). The production of cider is heavily dependent on the chemical properties of sulphite, both as a means of fermentation control and product preservation; the manufacturing process is briefly considered here. Large-scale manufacture of cider uses apples grown expressly for that purpose. Selected cider-apple varieties are

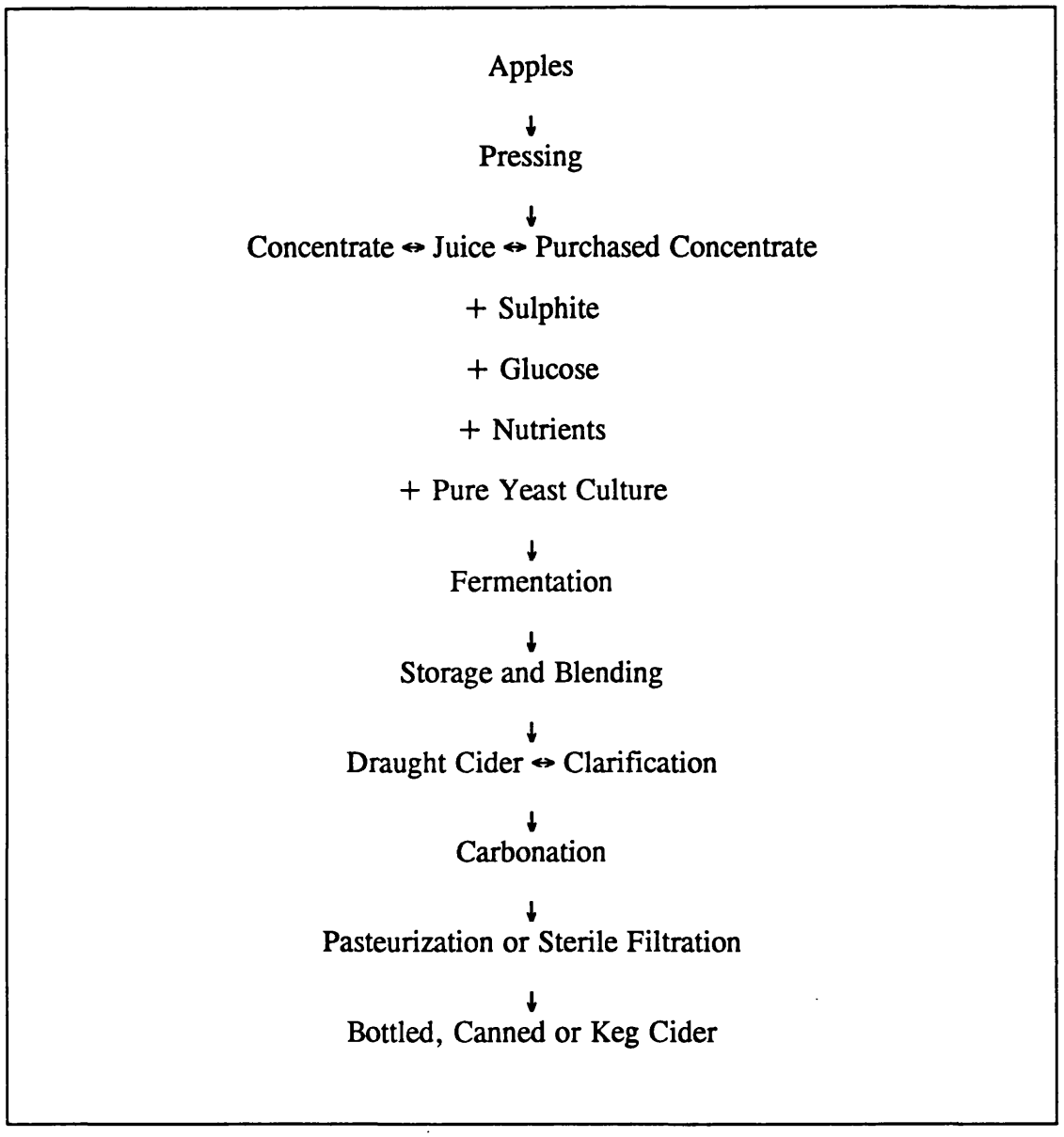


Figure 2. Flowchart for the manufacture of cider.

grown intensively on bush trees planted in herbicide-treated strips. Modern ciders are fermented mainly from the juices of bittersweet apples; these are characterized by low titratable acidity (less than 0.45 %, w/v) and high contents of phenolic compounds (greater than 0.20 %, w/v). Phenolic compounds impart bitterness and astringency to the flavour of a cider, and inhibit enzymic solubilization of fruit pectin so that bittersweet apples can be harvested and stored without excess damage

(Beech, 1972). The British apple harvest occurs in a short season spanning October to December. Cider apples are harvested mechanically; the trees are shaken to make the apples fall, and they are either caught or removed from the ground by vacuum. Fruit is transferred, in open container trucks, to cider factories where it is tipped into outdoor silos. Fully-ripe apples are carried into the factory by flotation, which serves to wash the fruit and to separate sound apples from rotten ones. Washwater must be strained and chlorinated continuously for it to have any cleansing effect. Archimedean screws or elevator bands lift apples from the washwater and into the processing plant, where the apples are pressed. The cider-making process is summarized in Figure 2. Automatic hydraulic presses are favoured in modern cider factories; they are more efficient and minimally labour-intensive in comparison to old-fashioned rack and cloth presses. Residual apple tissue (pomace) is used for production of pectin, or sold as cattle feed. Fresh apple juice produced in excess of needs can be concentrated for storage. Production of fully concentrated apple juice involves first reducing the volume of fresh juice by 10 - 15 %, by flash treatment at 95°C. Pectin is then removed and the juice clarified, before evaporating six or eight times under vacuum at 45°C, to above 70 % soluble solids or a specific gravity of 1.320. Concentrated juice is stored at 5°C under a blanket of carbon dioxide and can be used for fermentations outside the apple-processing season (Beech, 1993).

Freshly-pressed juice or apple-juice concentrate reconstituted with water is treated with sulphite. Ideally, fresh juice should be treated immediately after pressing and this can be achieved by mixing appropriate volumes of sodium metabisulphite solution with the flow of apple juice departing the press. Alternatively, apple juice may be transferred to epoxide resin-lined tanks and treated with sulphite; solutions of sodium metabisulphite or gaseous sulphur dioxide are common forms of sulphite addition. The optimum concentration of sulphite which should be added to a particular juice is dictated by the pH value (Table 3).

Apple Juice pH Value	Sulphite Concentration (p.p.m.)
3.0 - 3.3	75
3.3 - 3.5	100
3.5 - 3.8	150
3.8 - 4.0	200
4.0 and above	acidify to pH 3.8 and add 150 p.p.m.

Table 3. Appropriate concentrations of sulphite for treatment of apple juice prior to fermentation. From Beech (1993).

Following sulphite addition, juice is stored overnight for equilibration of the preservative to occur. When sulphite is added in this way, part of it combines more or less rapidly with various carbonyl compounds, some of which will be derived from apple juice, and some produced by contaminating organisms or fermentation yeasts. Acetaldehyde is recognized as a major sulphite binding compound in apple juices and ciders (Table 1). The most common yeasts present in fresh apple juice are of the genera *Candida*, *Torulopsis*, *Rhodotorula*, *Sporobolomyces*, *Kloeckera*, *Hansenula* and *Cryptococcus* (Beech and Carr, 1977). Sulphite suppresses these as well as the bacterial population of apple juice, and at the same time prevents excessive oxidation of juice components. The overall result of adding sulphite is production of fresh-flavoured ciders free from taints (Beech, 1972).

Legislation in the United Kingdom refers to total sulphur dioxide contents of ciders. Currently, the legal limit for sulphite in cider is 200 p.p.m. (3.12 mM; National Association of Cider Makers, 1988). The concern over safety of

preservatives in food and beverages, discussed in the previous section, has led to an ongoing debate about the maximum concentrations of sulphite which should be permitted in ciders. It is conceivable that, at some future time, the maximum permitted concentration may be lowered; this possibility was, in part, a stimulus for the present work.

Juice containing equilibrated sulphite may be adjusted to a standard specific gravity with sugar before fermentation is initiated. Also, nutrients such as thiamin, ammonium carbonate and ammonium phosphate may be added at this stage. Previously, fermentations were allowed to occur naturally with wild *Saccharomyces* species gaining dominance in the fermenter. Nowadays, a consistent product is demanded and apple juice is inoculated with pure cultures of *Saccharomyces cerevisiae* which have usually been grown in sterilized apple juice. The desirable properties of so-called pitching yeasts are summarized in Table 4. For a pitching yeast to dominate a fermentation, a minimum concentration of about 5 x 10^6

Good fermentation speed.
Appropriate resistance to sulphite, alcohol and low pH values.
Efficient utilization of sugars.
Low oxygen requirements.
Minimum production of hydrogen sulphide and sulphite-binding compounds.
Low requirement for vitamins and fatty acids.
Non-foaming.
Settle out rapidly.
Production of required pattern of flavour compounds.

Table 4. Desirable properties of pitching yeasts.

cells ml^{-1} of treated apple juice is necessary (Rankine and Lloyd, 1963). Almost immediately after the onset of fermentation all free sulphite becomes bound by carbonyl compounds produced by the organisms present. Controlled-condition fermenters which have become established in beer and lager industries are on the whole not used in cider-making. The bulk of cider fermentations are carried out in very large steel tanks, up to one million gallons in capacity, which are lined internally with an epoxide resin coating (Beech, 1972). Fermentations are influenced by ambient conditions; in very cold weather, yeasts will grow slowly if at all, whereas in warmer months temperature regulation of large fermentation volumes is extremely difficult. The exothermic nature of fermentation can lead to temperatures at which yeast activity declines (greater than 30°C), and undesirable bacterial growth is encouraged (Beech, 1976). Fermentations are normally allowed to proceed to dryness, defined by a sugar content of less than 10 g l^{-1} (Beech, 1976).

Following cessation of fermentation, ciders are left for a time for the yeast to settle out, and are then decanted into storage vats. During storage, new ciders may be blended with apple juice and/or new and older ciders to achieve the established flavour of the product. Whilst awaiting packaging, the blended cider may undergo malo-lactic fermentation, and this necessitates addition of synthetic malic acid to restore acidity to the product. The bacteria responsible for malo-lactic fermentation include species of *Lactobacillus*, *Leuconostoc* and rarely *Pediococcus* (Amerine and Kunkee, 1968; Beech and Davenport, 1983).

Blended ciders are clarified by centrifugation or filtration. A further addition of sulphite is made at this stage to combine with dissolved and headspace oxygen in the packaged product (Beech and Davenport, 1983); bottled products contain up to 50 p.p.m. free sulphite. Ciders may be artificially sweetened with sugars or apple juice; some ciders are artificially carbonated beforehand. The product may be sterilized before packaging by flash-pasteurization or filtration, with

both processes followed by aseptic filling. Alternatively, hot filling may be employed, or the packaged product pasteurized.

MICROBIOLOGICAL PROBLEMS IN CIDER MANUFACTURE.

The acidic pH value and presence of sulphite in apple juice and cider minimize spoilage due to bacteria. Disorders such as 'oiliness', 'ropiness' and 'mouse' due to bacteria now rarely occur in factory-produced ciders (Beech and Davenport, 1983). Storage of dry ciders and sweetening only before bottling has overcome the formerly disastrous outbreaks of 'cider sickness' caused by *Zymomonas* species. Exclusion of air prevents growth of acetic-acid bacteria (Beech and Carr, 1977).

Yeasts of the genus *Brettanomyces* can grow in ciders made with insufficient sulphite treatment and access to air, for example headspace air. They produce alkyl-substituted tetrahydropyridines, the source of so-called mouse taint (Heresztyn, 1986). This is a particular problem in the production of canned cider which does not contain free sulphite, as this degrades the can-linings, and in which the total sulphite concentration does not exceed 25 p.p.m. (P. Dockerty, personal communication). The problem is combatted by producing cider for canning by rapid fermentation using a large pitching-yeast inoculum, and where possible using sterilizable fermentation vessels.

Growth of any micro-organisms in a packaged product is a most horrible, and potentially expensive, scenario for a beverage manufacturer. In a process which depends upon the antimicrobial properties of sulphite, it is inevitable that the ecosystem so produced will select for an organism capable of tolerating high concentrations of this compound. *Saccharomycodes ludwigii* is exceptionally resistant to sulphite (Beech and Carr, 1977; Stratford *et al.*, 1987) and, if through failure of pasteurization or some other shortfall, it reaches the packaged product

then it is capable of growth. For example, *S'codes ludwigii* will grow at the bottom of cider-filled bottles, and as soon as the bottle is inverted a snow flake-like shower of clumps appear. It would be an extremely difficult task to eradicate *S'codes ludwigii* from the cider factory, and the organism is generally considered to be a serious threat to cider-making.

ANTIMICROBIAL ACTIVITY OF SULPHITE.

Sulphite is more effective in inhibiting bacterial and mould contamination than that caused by yeasts. Overall, the selective nature of sulphite enhances its value in control of undesirable fermentation and contamination in cider-making, and only under exceptional circumstances, such as infection with *S'codes ludwigii*, is the preservative ineffective.

Free molecular sulphur dioxide has generally been considered to be the only active antimicrobial form of sulphite. Molecular sulphur dioxide is more than 1000 times as active as bisulphite or sulphite anions against *Escherichia coli*, 500 times more effective against yeasts and 100 times more effective against *Aspergillus niger* (Rehm and Wittman, 1962). However, bound sulphite should not be considered to be an irretrievable form of sulphur dioxide. Combination of sulphite with carbonyl compounds is reversible, to a greater or lesser extent, depending on their respective equilibrium constants. Reports of the antimicrobial properties of bound sulphite suggest that free sulphur dioxide is released either by the bound complex being metabolized or simply by virtue of the dynamic equilibrium in existence between the bound and free species (Beech and Thomas, 1985). Warth (1985) observed that the growth of several yeast species remained inhibited after all free sulphite had become bound. Stratford and Rose (1985) exposed a strain of *Sacch. cerevisiae* to a pyruvate-sulphite complex, prepared from either ^{14}C-labelled pyruvate or ^{35}S-labelled sulphite. They found that radioactively-labelled sulphite was accumulated

by organisms more quickly than pyruvate, strongly suggesting that dissociation of the complex takes place before its components are transported by organisms. This challenges the belief that bound forms of sulphite are devoid of antimicrobial activity.

INTERACTIONS OF *SACCHAROMYCES CEREVISIAE* AND *SACCHAROMYCODES LUDWIGII* WITH SULPHITE.

The type species of the genus *Saccharomyces* is *Sacch. cerevisiae*. Normally, the yeast reproduces asexually by multilateral budding. Cells are globose (5 - 10 x 5 - 12 μm), ellipsoidal or cylindrical (3 - 9.5 x 4.5 - 21 μm; Barnett *et al.*, 1983). Cells of *S'codes ludwigii* are apiculate or elongate, and are generally larger (4 - 7 x 8 - 23 μm) than *Sacch. cerevisiae* (Barnett *et al.*, 1983). *Saccharomycodes ludwigii* reproduces by bipolar budding on a broad base (bud fission) and, in the process of budding, cells resemble bowling pins. *Saccharomyces cerevisiae* and *S'codes ludwigii* differ in the sugars which they can utilize, with the former yeast being generally more versatile in this respect (Table 5). *Saccharomyces cerevisiae* is generally considered to be one of the more sulphite-resistant species of yeasts (Ough, 1983). However, *S'codes ludwigii* is considerably more resistant to this compound than *Sacch. cerevisiae*. Stratford *et al.* (1987) found that *Sacch. cerevisiae* TC8 was unable to grow in the presence of 1.56 mM sulphite, whereas *S'codes ludwigii* TC10 was able to grow in the presence of sulphite concentrations up to and including 7.8 mM. The remainder of this Introduction will focus on the effects of sulphite on *Sacch. cerevisiae* and *S'codes ludwigii*, and particular attention will be given to factors which could explain their markedly different tolerances of sulphite. Unfortunately, there is often a scarcity of information pertaining to *S'codes ludwigii*, and cross-references with other yeast species, most notably sulphite-tolerant *Zygosacch. bailii* (Pilkington and Rose,

	Saccharomyces cerevisiae	*Saccharomycodes ludwigii*
Glucose	+	+
Galactose	+ -	-
Sucrose	+ -	+
Maltose	+ -	-
Raffinose	+ -	-
Lactose	-	-

Table 5. Sugars which support aerobic growth and fermentation of *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii*. Ability to utilize a sugar denoted '+', inability to do so '-' and where utilization differs amongst strains '+ -'. From Barnett *et al.* (1983).

1988), have to be included. Similarly, references to weak-acid preservatives such as benzoate and sorbate are necessary where little information is available for sulphite-induced effects on yeasts.

TRANSPORT OF SULPHUR DIOXIDE INTO THE YEAST CELL.

Yeast cells, incubated with radioactively-labelled sulphite at a range of pH values, accumulate amounts of radioactivity in direct proportion to the concentrations of molecular sulphur dioxide present. Yeast cells incubated at the same range of pH values, but with total sulphite concentrations adjusted so that the same concentrations of molecular sulphur dioxide are present at each pH value,

accumulate the same amount of radioactivity. Such an experiment (Macris and Markakis, 1974) indicated that, in an aqueous solution of sulphite, only undissociated sulphur dioxide is transported into cells of *Sacch. cerevisiae*. This was confirmed by Stratford and Rose (1986) when they recorded a correlation between the rate of uptake of sulphite by *Sacch. cerevisiae* and the calculated concentration of molecular sulphur dioxide present in solutions at different pH values.

Further experiments by Macris and Markakis (1974), to elucidate the mechanism of sulphite uptake, were interpreted as indicating an active carrier-mediated system. However, it is now generally accepted that *Sacch. cerevisiae* accumulates sulphite by passive diffusion of molecular sulphur dioxide; Stratford and Rose (1986) provided the most convincing evidence for this. They (Stratford and Rose, 1986) obtained near-vertical Woolf-Hofstee plots (Hofstee, 1959) for uptake of sulphite in *Sacch. cerevisiae* at pH 3.0 and 4.0; initial uptake rates were found to rise in proportion to increases in molecular sulphur dioxide concentration. Neither plot was perfectly vertical, so that kinetic constants could be derived from them; values for K_M were 3.2 mM and 0.11 mM at pH 3.0 and 4.0, respectively. These K_M values are far in excess of sulphite concentrations which would have killed *Sacch. cerevisiae* under the conditions of the experiments. Additional evidence for the lack of involvement of an active process was provided by the failure of carbonyl cyanide *m*-chlorophenylhydrazone and 2,4-dinitrophenol, both of which are known to dissipate proton-motive force and inhibit protein-mediated transport systems, to affect initial velocities of sulphite accumulation. Furthermore, exclusion of glucose from the reaction mixture did not affect sulphite uptake, nor did addition of the glycolytic inhibitor 2-deoxy-D-glucose. No effects of pH value on sulphite accumulation, other than predicted by changes in sulphite concentration,

were observed. Experiments such as these have demonstrated that sulphite is accumulated by free diffusion of molecular sulphur dioxide in *S'codes ludwigii* (Stratford *et al.*, 1987), and also in *Zygosacch. bailii* (Pilkington and Rose, 1988).

In all of the studies described above which employed Woolf-Hofstee plots, there were deviations from the vertical at very low concentrations of molecular sulphur dioxide. This suggests that, at low concentrations of sulphite, a facilitated transport system may operate, possibly to transport the bisulphite anion. The activity of such a system would become masked at higher concentrations of molecular sulphur dioxide (Pilkington and Rose, 1988).

In both *Sacch. cerevisiae* and *S'codes ludwigii*, plasma membranes act as selective barriers to free diffusion of sulphite. For this reason, the relative structure and fluidity of the plasma membrane most probably affects transport. Indeed, initial velocities of sulphite accumulation are greater with *S'codes ludwigii* than with *Sacch. cerevisiae* (Stratford *et al.*, 1987). This is of particular interest in relation to the report that *S'codes ludwigii* is richer than *Sacch. cerevisiae* in $C_{18:1}$ phospholipid fatty-acyl residues (Kaneko *et al.*, 1976). More than 50 % of the cellular lipids of *S'codes ludwigii* have chain lengths of greater than 18 carbon atoms, whereas more than 50 % of the lipids of *Sacch. cerevisiae* have chain lengths of less than 16 carbon atoms. Stratford *et al.* (1987) hypothesized that *S'codes ludwigii*, being richer in unsaturated phospholipid fatty-acyl residues, would have a greater membrane fluidity than *Sacch. cerevisiae* and argued that this facilitates diffusion of sulphur dioxide across the plasma membrane. However, studies of sulphite accumulation in *Zygosacch. bailii* with respect to phospholipid fatty-acyl unsaturation and chain length, have indicated precisely the opposite correlation; permeability coefficients were greater the lower the amount of unsaturation in plasma-membrane phospholipids (Pilkington and Rose, 1989).

ROLE OF INTRACELLULAR pH VALUE IN SULPHITE ACCUMULATION.

Saccharomyces cerevisiae and *S'codes ludwigii* accumulate sulphite initially very rapidly, reaching a plateau concentration after about five minutes exposure. After this time, intracellular sulphite concentrations are many times greater than in suspension (Stratford and Rose, 1986; Stratford *et al.*, 1987). Yeast intracellular pH values, if such can be comprehended, are usually maintained at just below neutrality, irrespective of minor fluctuations in extracellular pH values; Table 6 lists examples of intracellular pH values for selected yeast species.

Molecular sulphur dioxide, upon entering a yeast cell, dissociates to form the familiar pH-dependent equilibrium, and normal intracellular pH values favour formation of bisulphite and sulphite anions. Sulphite will continue to enter a yeast cell until intracellular and extracellular concentrations of molecular sulphur dioxide are in equilibrium. Therefore, total intracellular concentrations of sulphite have to be considerably higher than those outside the cell for there to be sufficient molecular sulphur dioxide to maintain the equilibrium. A high intracellular pH value relative to that extracellularly results in intracellular sulphite concentrations in excess of those external to the cell, and it follows that the larger the pH differential between these environments, the greater will be the concentration of sulphite accumulated by a yeast.

There is little in the literature which is relevant to the relationship between sulphite accumulation and intracellular pH value in *Sacch. cerevisiae* and *S'codes ludwigii*. Pilkington and Rose (1988) reported measurements of intracellular pH values in *Sacch. cerevisiae* and *Zygosacch. bailii*, with respect to sulphite accumulation, but did not comment greatly on the relationship between these components. Warth (1977) did not find a direct relationship between intracellular pH value and accumulation of benzoic, sorbic, butyric or acetic acids by *Zygosacch. bailii*. He (Warth, 1977) found that intracellular pH values calculated from

	Intracellular pH Value
Saccharomyces cerevisiae	6.35 [CD], 5.81 [K], 7.20 [W88], 6.90 [W88], 6.70 [P], 6.60 [P]
Saccharomycodes ludwigii	7.10 [W88]
Zygosaccharomyces bailii	5.80 - 6.10 [W77], 5.70 [CK], 6.05 [CK], 6.90 [W88] 6.50 [P], 6.40 [P]
Schizosaccharomyces pombe	6.90 [W88]
Kloeckera apiculata	6.80 [W88]
Hansenula anomala	7.10 [W88]

Table 6. Intracellular pH values of certain yeast species. Values quoted apply to actively growing cells, but measurements were made under different conditions, are strain-dependent and a range of measurement techniques were employed; therefore individual publications should be sought for details. Values are quoted from Conway and Downey (1950; CD), Warth (1977; W77), Krebs *et al.* (1983; K), Cole and Keenan (1987; CK), Pilkington (1988; P) and Warth (1988; W88).

$pH = pKa + \log_{10} [A^- / HA]$, where HA = undissociated preservative and A^- = preservative anion, were 0.27 - 0.50 units lower than pH measurements made by a freeze-thaw method (Conway and Downey, 1950). Expressed in a different way, *Zygosacch. bailii* accumulated much lower amounts of weak acid preservatives than were predicted from intracellular pH measurements. It was suggested that this discrepancy may have been due to compartmentalization within organisms, binding of preservative to cell components, buffering action of cell walls or permeability of

plasma membranes to preservative anions. Warth (1977) concluded that only the existence of a significant permeability of organisms to preservative anions would cause the discrepancy. It is probable that such findings with lipophilic weak acids are relevant to sulphite accumulation, as there are only small differences in the relative effectiveness of these preservatives between species; for example, species tolerant of benzoic acid tend to be tolerant of other preservatives, including sulphite (Warth, 1988).

Cole and Keenan (1987) were critical of the freeze-thaw method of intracellular pH value-measurement used by Warth (1977). They (Cole and Keenan, 1987) employed propionic acid accumulation to measure intracellular pH values of *Zygosacch. bailii* and, in the presence of weak-acid preservatives, recorded lower values than did Warth (1977). The concentration of benzoic acid accumulated intracellularly was identical to the value expected from its pKa value, and external and internal pH values (Cole and Keenan, 1987). It should be pointed out, however, that Cole and Keenan (1987) cited the publication of Conway and Downey (1950) as evidence of a poor correlation between freeze-thaw and weak-acid accumulation methods of intracellular pH measurement; Krebs *et al.* (1983) cited the same publication as providing a good correlation between the two methods in question.

Intracellular pH values of yeasts vary with culture age. Once again, there is no relevant information available for *S'codes ludwigii* regarding this topic, but age-dependent intracellular pH values in *Sacch. cerevisiae* and *Zygosacch. bailii* have been reported. Tracey Holland (unpublished data) found that intracellular pH values of *Sacch. cerevisiae* Y185 and those of *Zygosacch. bailii* NCYC 1427 were lowest during the stationary phase of growth. These values could, in turn, be correlated with accumulation of relatively small concentrations of benzoic acid. In addition, intracellular pH values were found to be most alkaline during early-exponential phase when compared to other growth phases.

Organisms exhibit different capacities to regulate their cytoplasmic pH values; *ie.* they possess different intracellular buffering capacities. In many organisms, intracellular pH values may vary by 0.1 unit, whereas in others much larger changes in internal pH value have been observed (Booth, 1985). Accumulation of sulphite by yeast cells causes a decrease in intracellular pH value to different extents in different organisms (Pilkington and Rose, 1988). Tracey Holland (unpublished data) observed that intracellular pH values of *Sacch. cerevisiae* were more prone to fluctuation than those of *Zygosacch. bailii*, and suggested that the latter yeast might possess a more efficient mechanism for maintaining cytoplasmic pH values. It is an unfortunate fact that the literature is not well-blessed with information about the relationship between sulphite accumulation and intracellular buffering capacities, despite the importance of the latter component in influencing to what extent intracellular pH values are regulated. Suffice to say, intracellular buffering capacities are not identical in different organisms, and an investigation into the significance of this, with regard to preservative-resistance, is long overdue.

TOXIC EFFECTS OF SULPHITE IN YEAST.

Highly reactive sulphite molecules are faced with a massive array of potential chemical reactions in the shape of a yeast cell. It would seem improbable that the cause of sulphite-induced yeast inhibition could be pin-pointed. Despite the apparent magnitude of the task, there has been a search for the most sulphite-sensitive molecules. The cellular 'targets' for sulphite are discussed here.

Much of the research into sulphite-induced toxicity in yeasts has been confined to intracellular reactions. Very little attention has been given to the potential for direct disruption or inhibition, by sulphite, of cell-wall or plasma-membrane components. It is possible that proteins present in the cell wall, and

those exposed on the outside of the plasma membrane, are the main targets for sulphite, external to the cytosol. With regard to effects on plasma membranes of yeast cells, it has been shown that sulphite inhibits accumulation of the amino acids lysine and arginine in *Sacch. cerevisiae* (Stratford, 1983). Amino-acid accumulation could, in theory, be inhibited by sulphite-induced denaturation of transport proteins, exposed on the outer surface of the plasma membrane. Alternatively, sulphite could cause dissipation of the proton-motive force across the membrane, thereby inhibiting active transport of solutes; however, information gathered in Holzers' laboratory disagrees with this, and the evidence against will be presented later in this section.

A mechanism by which exposed plasma-membrane proteins would be disrupted, as described above, was outlined by Anacleto and van Uden (1982). They proposed a three-stage mechanism to describe events leading to cell death in *Sacch. cerevisiae*, as follows: (i) sulphite binds to cell-surface receptors causing (ii) a change of activity of the receptors and (iii) the consequences of this result in loss of viability. Two potential receptors were proposed. Sulphite may bind to exposed plasma-membrane proteins involved in structure or solute-transport, and in doing so lower the free energy of denaturation, such that protein structure is disrupted. A leaky membrane would be produced and subsequent cell death would result from loss of intracellular molecules. They (Anacleto and van Uden 1982) did not speculate on the identity of this receptor, and did not provide hard evidence to substantiate their theory. However, an observation by Warth (1989) may provide oblique evidence. He (Warth, 1989) discovered that relatively high concentrations of propionic acid increased the rate of uptake of the compound by *Zygosacch. bailii*, and proposed that the tendency to an increased rate was caused by disruption of membrane structure. Although accumulation of propionic acid by yeast can be considered to be analogous to sulphite accumulation (Warth, 1989), sulphite-induced membrane disruption has not been reported. The second receptor which Anacleto and van Uden (1982) proposed was membrane-bound ATPase. According

to their theory, binding of sulphite to ATPase complexes leads to uncontrolled hydrolysis of ATP. Cell death results from cellular ATP depletion and subsequent interruption of ATP-dependent processes. Sulphite-induced ATP depletion does indeed occur, and persuasive evidence of its role in cell death has been provided by Holzers' laboratory. On reviewing their evidence, the emphasis of this passage shifts from cell-wall and plasma-membrane reactions to intracellular reactions of sulphite.

Schimz and Holzer (1979) observed that, prior to death of sulphite-treated *Sacch. cerevisiae*, there was a rapid decrease of cellular ATP and an increase in the level of inorganic phosphate; the content of ADP remained essentially constant. Contents of CTP, GTP and UTP in sulphite-treated cells showed parallel changes to those of ATP (Hinze and Holzer, 1986). It was concluded that gross depletion of cellular ATP is probably the major cause of cell death due to sulphite (Schimz, 1980). Depletion of ATP was found to be reversible, and cell death could be prevented if cells were exposed to sulphite for periods of less than one hour. Further to these studies, the effects of sulphite on enzymes involved in glucose catabolism (glycolysis) were studied. It appears that the principal cause of ATP depletion is inhibition of glyceraldehyde-3-phosphate dehydrogenase (Figure 3). Hinze and Holzer (1985) provided evidence for this by assaying fifteen enzyme activities in extracts from *Sacch. cerevisiae* incubated with sulphite. They discovered that glyceraldehyde-3-phosphate dehydrogenase was inhibited to the greatest extent, and attributed sulphite-induced cellular-ATP depletion to this. Furthermore, Maier *et al.* (1985) reported that, on addition of 5 mM sulphite to suspensions of *Sacch. cerevisiae* at pH 3.6, glyceraldehyde-3-phosphate activity was decreased, *in vivo*, by 98 %. Alcohol dehydrogenase was inhibited to a lesser extent (80 %), and NAD^+-glutamate dehydrogenase by 60 %.

The fact that inhibition of ATP production by sulphite is caused primarily by inhibition of substrate level-phosphorylation, and not through inhibition of respiratory chain-phosphorylation, was elegantly demonstrated by Hinze and

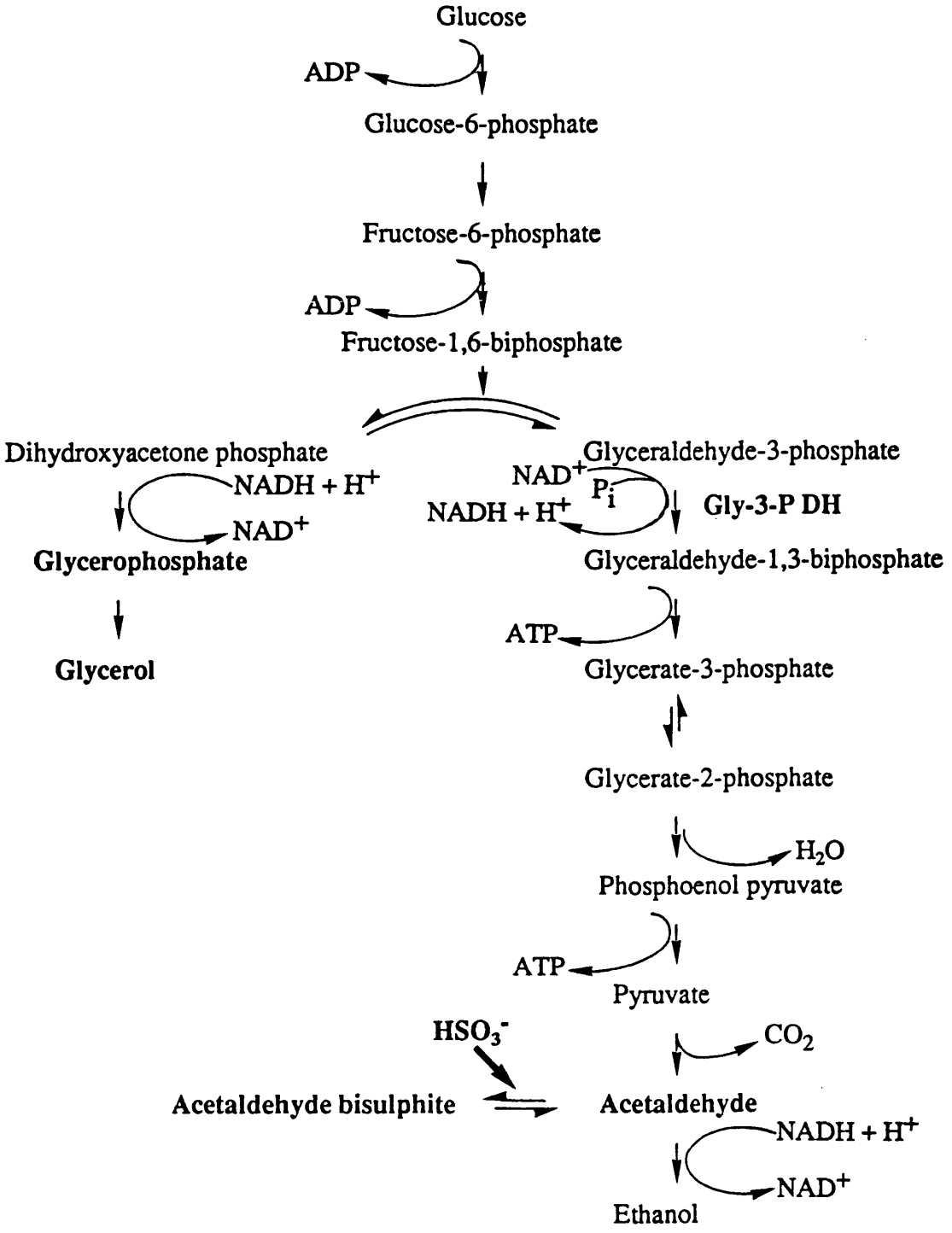


Figure 3. Flowchart representation of glycolysis. Sulphite inhibits glyceraldehyde-3-phosphate dehydrogenase (Gly-3-P DH), and binds to acetaldehyde (which leads to production of glycerol); effects of sulphite are shown in bold letters.

Holzer (1986). They exposed a *Sacch. cerevisiae* mutant (pet 936), which is defective in the mitochondrial F_1-ATPase and therefore defective in respiratory chain-phosphorylation, to sulphite. The defective yeast produced the same rate of decrease of ATP content after sulphite addition as did the wild-type yeast, indicating that inhibition of yeast energy-generating metabolism by sulphite is confined to glycolysis. Thus there is no proof for the theory of Anacleto and van Uden (1985) that sulphite inhibits membrane-bound ATPase. Although the activity of membrane-bound ATPase can be decreased by sulphite action *in vitro*, experiments with the mutant pet 936 indicate that this has little significance *in vivo* (Maier *et al.*, 1986).

In addition to inhibiting glycolysis as described above, the reaction of sulphite with acetaldehyde to form a stable hydroxysulphonate contributes to overall ATP depletion (Hinze and Holzer, 1986; Figure 3). In yeast alcoholic fermentations, pyruvate, the end product of glycolysis, is decarboxylated to acetaldehyde by the enzyme pyruvate decarboxylase. Acetaldehyde serves as an electron acceptor and is reduced by alcohol dehydrogenase to ethanol. However, when sulphite is present, the bisulphite anion combines with acetaldehyde which cannot then be reduced by NADH. This blocks regeneration of NAD^+ which is needed for oxidation of glyceraldehyde 3-phosphate. Under such circumstances, another pathway for oxidizing NADH is utilized. Dihydroxyacetone phosphate is reduced by NADH in the presence of alcohol dehydrogenase. As a consequence, glycerophosphate (in turn converted to glycerol) is formed in amounts equivalent to the quantity of acetaldehyde trapped and carbon dioxide released. There is no net energy gain for the organism by the sulphite-induced pathway.

There are other potentially important intracellular reactions of sulphite. Glucose and dihydroxyacetone phosphate combine with sulphite, thereby inhibiting the glycolytic pathway. Binding of sulphite with other cellular components such as oxaloacetate, glutaric acid and NAD^+ has been demonstrated. Cleavage of thiamin is known to occur, and has already been mentioned in this text. The multitude of

intracellular reactions in which sulphite could participate would be expected to make contributions to inhibition of energy-generating pathways of micro-organisms.

RESISTANCE OF *SACCHAROMYCES CEREVISIAE* AND *SACCHAROMYCODES LUDWIGII* TO SULPHITE.

Resistance to the antimicrobial action of sulphite by yeasts appears to be an inherent property of the species or strain, and is not induced by the presence of sulphite. There is one report, however, which questions this. In experiments with twelve yeast strains, including *Sacch. cerevisiae* and *S'codes ludwigii*, Warth (1985) observed that many growth curves did not show an increase corresponding to depletion of free sulphite. He (Warth, 1985) suggested that, once adapted to grow in sulphite, these cells were not greatly inhibited by its presence. This report is unique and no corroborative data are available. Indeed, King *et al.* (1981) stated categorically that yeasts do not acquire tolerance to sulphite through exposure to the preservative.

In media containing sulphite, both *Sacch. cerevisiae* and *S'codes ludwigii* show an increase in the lag phase of growth and a loss of colony-forming ability (Stratford *et al.*, 1987). These effects are only observed to take place with *S'codes ludwigii* at much higher concentrations of sulphite than those required to inhibit *Sacch. cerevisiae*. Stratford *et al.* (1987) found that *S'codes ludwigii* was able to grow at sulphite concentrations five times those which killed *Sacch. cerevisiae*. In experiments with *Sacch. cerevisiae*, *S'codes ludwigii*, *Zygosacch. bailii* and *Schiz. pombe*, Warth (1985) found that sulphite did not greatly inhibit growth rates and had almost no effect on cell yields, but did produce extended lag phases; *S'codes ludwigii* was exceptional in showing markedly shorter lag times than the other yeasts. Clearly, the stratagem which *S'codes ludwigii* employs to resist the action

of sulphite is superior to that of *Sacch. cerevisiae*. Stratagems which could enhance survival fall into three categories:

(i) Resistance of targets to inactivation by sulphite.

(ii) Inactivation of sulphite before it can reach target molecules.

(iii) Decrease of the concentration of sulphite which reaches target molecules.

It is not inconceivable that slight differences in structure or conformation of key molecules could provide a degree of resistance to sulphite in some yeast species, but not others. The major mechanism by which sulphite affects yeasts intracellularly is considered to be by inhibition of glyceraldehyde-3-phosphate dehydrogenase, as already described. It is possible that access to this enzyme in *S'codes ludwigii* is less readily achieved than in *Sacch. cerevisiae*. Currently, there is no evidence to suggest that compartmentalization within cells differs between *Sacch. cerevisiae* and *S'codes ludwigii*. It would not be unreasonable to speculate that the glyceraldehyde-3-phosphate dehydrogenase of *S'codes ludwigii* is inhibited to a lesser extent than that of *Sacch. cerevisiae*; *ie.* target sites for sulphite action are not equally susceptible in all yeast enzymes.

Production of acetaldehyde which binds with sulphite and, arguably, inactivates the preservative, has been reported for both *Sacch. cerevisiae* and *S'codes ludwigii* (Stratford *et al.*, 1987). A hypothesis which explains sulphite resistance partly in terms of relatively low intracellular pH values has been proposed (Stratford *et al.*, 1987). A new hypothesis, which takes into account intracellular buffering capacities along with intracellular pH values, is given in this thesis.

Sulphite-Induced Binding-Compound Production.

In the presence of sulphite, both *Sacch. cerevisiae* and *S'codes ludwigii* produce acetaldehyde, which binds avidly with sulphite (Table 1), a reaction which would seem to fulfil stratagems (ii) and (iii) of the categories given above. Warth

(1985) attributed the lack of effect of sulphite on overall growth rates and cell yields of a range of yeast species, including *Sacch. cerevisiae* and *S'codes ludwigii*, to the production of sulphite-binding compounds; the major part of growth occurred after free sulphite was bound.

According to Neuberg's second form of fermentation, acetaldehyde and glycerol are formed in equimolar amounts and in quantities equivalent to the concentration of sulphite initially present (Mahler and Cordes, 1966; Figure 3). However, Stratford *et al.* (1987) reported that *S'codes ludwigii* produced acetaldehyde in amounts that were 25 % in excess of those required to bind all of the sulphite present; *Sacch. cerevisiae* produced acetaldehyde with only a 4 % excess. Such a remarkable over-production of the sulphite-binding compound acetaldehyde, by a yeast noted for its resistance to sulphite, was considered unlikely to be coincidental. However, the findings of Pilkington and Rose (1988) were in general agreement with Neuberg; acetaldehyde and glycerol were produced by strains of *Sacch. cerevisiae* and *Zygosacch. bailii* in amounts equimolar to the concentrations of sulphite present. In this study (Pilkington and Rose, 1988), the correlation between relatively high sulphite tolerance and a capacity to produce large concentrations of acetaldehyde held true, although over-production of acetaldehyde was not reported.

Pyruvate, which combines with reasonable stability with sulphite (Table 1), does not seem to be important in the resistance of these yeasts, and concentrations of this compound are not significantly increased by the presence of sulphite (Stratford *et al.*, 1987; Pilkington and Rose, 1988).

Influence of Intracellular pH Values and Buffering Capacities in Sulphite Resistance.

It is very likely that the stresses of anion accumulation and pH value-maintenance are important in determining cell inhibition and death due to sulphite.

Physiological phenomena that allow organisms to cope with these stresses, or minimize them, will enhance their tolerance of sulphite.

It is established that molecular sulphur dioxide enters *Sacch. cerevisiae* and *S'codes ludwigii* by passive diffusion, and also that initial velocities of sulphite accumulation are greatest in the latter organism (Stratford and Rose, 1986; Stratford *et al.*, 1987). [As an aside, there seems to be no relationship between initial velocities of accumulation and sulphite resistance. In a survey of nine yeast species, Warth (1989) found that preservative-resistant yeasts generally had lower velocities of accumulation of the preservatives. This is the opposite correlation to that made by Stratford *et al.* (1987), detailed above]. However, in terms of net accumulation, *Sacch. cerevisiae* takes up considerably more sulphite than does *S'codes ludwigii*. Stratford *et al.* (1987) found that, in citrate buffer containing 1 mM total sulphite, *Sacch. cerevisiae* accumulated 19.1 nmol (μl intracellular fluid)$^{-1}$ compared to 6.2 nmol μl^{-1} by *S'codes ludwigii*. The initial velocities of accumulation may or may not be attributable to differences in plasma-membrane composition, but it is net accumulation which is of greatest importance. One explanation for the smaller accumulation of sulphite by *S'codes ludwigii* is that the organism may have a relatively low intracellular pH value in comparison to more sulphite-sensitive yeasts. The intracellular environment of a yeast cell favours formation of bisulphite and sulphite, and these anions are effectively trapped within a cell because of the impermeability of the plasma membrane to them. The concentration of sulphite which becomes trapped is dependent on intracellular pH values, and it follows that lower intracellular pH values result in smaller concentrations of sulphite accumulated. Stratford *et al.* (1987) postulated that, assuming equal intracellular buffering capacities, *S'codes ludwigii* would have a lower intracellular pH value than *Sacch. cerevisiae* and this in turn would decrease the concentration of sulphite reaching target molecules. Of the small amount of evidence available, there is none to back this hypothesis. Warth (1988) reported comparable values of intracellular pH value for *Sacch. cerevisiae* and *S'codes ludwigii* (Table 6). In contrast to an

intracellular pH value of 6.9 for *Sacch. cerevisiae* FRR 1298, that of *S'codes ludwigii* 1555 was found to be 7.1; of the two species, *S'codes ludwigii* 1555 was the more tolerant of benzoic acid. Similarly, in studies comparing intracellular pH values of *Sacch. cerevisiae* and *Zygosacch. bailii*, no correlation between sulphite tolerance and low intracellular pH value was made (Pilkington and Rose, 1988). Warth (1988) did not investigate intracellular buffering capacities, but Pilkington and Rose (1988) did comment that this factor might be important.

Passage of sulphite into a yeast cell acidifies the cytoplasm; Pilkington and Rose (1988) demonstrated that intracellular pH values of *Sacch. cerevisiae* and *Zygosacch. bailii* decrease upon accumulation of sulphite. However, it was also observed that decreases in intracellular pH values were not of the same magnitude in different yeast strains, indicating that intracellular buffering capacities were not equal. The theoretical importance of intracellular pH values in determining the extent to which an organism accumulates sulphite, discussed already, is central to the following considerations of intracellular buffering capacity. An organism with a relatively high intracellular buffering capacity would maintain a steady intracellular pH value despite accumulation of sulphite. In doing so the concentration of molecular sulphur dioxide, at a near neutral pH value, would remain comparatively small, and this would have the effect of drawing more sulphite into the cell. On the other hand, an organism with a relatively low intracellular buffering capacity would produce a rapid decline in intracellular pH upon acidification of the cytoplasm; creation of relatively small intracellular-extracellular pH differentials would decrease the concentration of sulphite accumulated. This brings into question the importance of yeast intracellular pH values in terms of the relationship between constant intracellular-environment maintenance and yeast survival. If an organism were able to tolerate low intracellular pH values, it would benefit from a small intracellular buffering capacity when in the presence of sulphite. Cole and Keenan (1987) reported that resistance of *Zygosacch. bailii* to benzoic and sorbic acids was at least partly due to its ability to tolerate chronic intracellular pH decreases; no

information of this type has been reported for either *Sacch. cerevisiae* or *S'codes ludwigii*. They (Cole and Keenan, 1987) also described a mechanism whereby, through decreasing protoplast volume and concentrating cellular components, buffering capacities of organisms are increased; the implication being that sulphite-tolerant organisms respond in this way more readily than sulphite-intolerant organisms. Pilkington (1988) disputed the importance of protoplast volume-changes in response to sulphite, and provided evidence to show that protoplast volumes remain constant despite accumulation of sulphite.

In order for a yeast cell to be able to recover from chronic decreases in intracellular pH value, its metabolism would have to continue functioning, or retain a capacity to resume function, in adverse acidic conditions. Organisms must therefore have a threshold value for intracellular pH value, below which resumption of normal metabolism would be impossible. It is not known whether this threshold value differs between sulphite-tolerant and sulphite-sensitive yeast species.

AIMS OF PROJECT.

The major aim of this project is to investigate the nature of sulphite resistance in yeasts and to try to improve our understanding of the mechanisms which impart extreme sulphite tolerance to *S'codes ludwigii* but not to *Sacch. cerevisiae*. The occurrence of sulphite-tolerant spoilage yeasts is also investigated to attempt to judge the consequences of a lowering of permitted sulphite concentrations in cider manufacture.

MATERIALS AND METHODS.

MATERIALS AND METHODS (PART A)

ORGANISMS.

The yeasts used were *Saccharomyces cerevisiae* AWRI 1A65, *Saccharomycodes ludwigii* BC1 and *S'codes ludwigii* TC10 (Stratford, 1983). Both *Sacch. cerevisiae* AWRI 1A65 and *S'codes ludwigii* BC1 were kindly provided by HP Bulmer Ltd. The strains were maintained at 4°C on slopes of M.Y.G.P. medium containing (l^{-1}) glucose (10 g), yeast extract (Lab M; 3 g), malt extract (3 g), mycological peptone (Lab M; 5 g) and agar (Lab M, No. 2; 20 g); (Wickerham, 1951). The medium was sterilized by autoclaving at 121°C for 15 min. Organisms were sub-cultured bi-monthly and, as a safeguard against loss, samples of each yeast were stored under liquid nitrogen.

EXPERIMENTAL CULTURES.

Yeasts were grown aerobically in medium containing (l^{-1}) glucose (20 g), ammonium sulphate (3 g), yeast extract (Lab M; 1 g), magnesium sulphate heptahydrate (30 mg) and calcium chloride dihydrate (30 mg). The medium was buffered with dipotassium hydrogen orthophosphate (13.4 g) and citric acid (12.9 g), adjusted to pH 4.0 with citric acid (Pilkington and Rose, 1988). Portions (1 l) of medium were dispensed into two-litre round, flat-bottomed flasks (Patching and Rose, 1969) which were plugged with cotton-wool and sterilized by autoclaving at 121°C for 15 min. Starter cultures (100 ml medium in 250 ml conical flasks) were inoculated with a pinhead of yeast from a slope culture and incubated at 30°C on an orbital shaker (200 rev. min^{-1}). One-litre portions of medium were inoculated with 1 mg dry wt. equivalent of yeast from a 24 h starter culture and incubated at 30°C with stirring (4 cm stirring bar; approx. 200 rev. min^{-1}).

Growth was monitored by relating absorbance (600 nm wavelength) of samples of culture to dry wt. of organisms by standard curves constructed for each yeast strain. Unless otherwise stated, organisms were harvested by centrifugation (10 000 *g*; 1 min; 4°C) in the mid-exponential phase of growth containing 0.70 mg dry wt. ml^{-1} for *Sacch. cerevisiae* AWRI 1A65, and 0.60 mg dry wt. ml^{-1} for *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10. In certain experiments, organisms were harvested from cultures in the early-exponential or stationary phases of growth containing 0.12 and 1.30 mg dry wt. ml^{-1}, respectively, for *Sacch. cerevisiae* AWRI 1A65 and 0.12 and 1.20 mg dry wt. ml^{-1}, respectively, for *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10. All centrifugation was carried out using a Sorvall RC5B refrigerated Superspeed Centrifuge (Du Pont, Wilmington, Delaware, USA).

MEASUREMENT OF SULPHITE TOLERANCE.

(a) MICROTITRE PLATES.

Harvested organisms were resuspended in sterile (121°C; 15 min) medium to give a 0.1 mg dry wt. ml^{-1} suspension. Using a multiple-channel pipette (Flow Laboratories, Rickmansworth, Hertfordshire, England), cell suspension (170 μl) was pipetted into each well of a microtitre plate, leaving one well empty to use as a reference blank. Appropriate concentrations of sodium metabisulphite (30 μl), freshly prepared in medium, were added to each well to give final concentrations of sulphite ranging between zero and 8 mM. Reference wells were filled with distilled water (200 μl). Prepared plates were covered and sealed in polythene bags with some moist tissue paper to minimize evaporation and incubated at 30°C on an orbital shaker (200 rev. min^{-1}). Using an automatic microtitre plate reader (Dynatech Laboratories Ltd., Billingshurst, Sussex, England), set at 600 nm wavelength, absorbances were measured after 6 h incubation, adjusting to zero against the reference wells. Plates were gently agitated for 2 min on a Titertek

Shaker (Flow Laboratories) prior to absorbance measurement as cells tended to settle at the bottom of wells.

(b) ONE-LITRE CULTURES.

Harvested organisms were resuspended in sterile medium (1 l) in two-litre round, flat-bottomed flasks to give a 0.5 mg dry wt. ml^{-1} suspension. Portions of sodium metabisulphite solution (10 - 200 mM) were included in the medium to give final sulphite concentrations ranging between zero and 8 mM. Cultures were incubated at 30°C with stirring and, at intervals up to 6 h, yeast growth was monitored by relating absorbance (600 nm wavelength) of samples of culture to dry wt. of organisms by standard curves constructed for each yeast strain. In addition, viable cells were estimated by serial dilution and plating of the dilutions onto M.Y.G.P. medium. Plates were incubated at 30°C for 48 h, after which time the number of colonies on each plate was recorded.

MEASUREMENT OF ACETALDEHYDE CONCENTRATIONS IN CULTURE.

Harvested organisms were resuspended to 0.5 mg dry wt. ml^{-1} in portions (1 l) of sterile medium in two-litre round, flat-bottomed flasks. Where appropriate, portions of sodium metabisulphite solution (10 - 200 mM) were included in the medium to produce sub-lethal concentrations of sulphite. At intervals up to 6 h, samples (1 - 2 ml) of culture were removed by drawing into a 2 ml syringe fitted with a 15 cm needle. The syringe was then attached to a filter assembly containing a membrane filter (0.45 μm pore size; 25 mm diameter) and filtrate was collected in a bijou bottle which was held on ice. Samples were assayed immediately using a commercially available kit (Boehringer Mannheim Ltd., Lewes, East Sussex,

England). Acetaldehyde was oxidized quantitatively in the presence of aldehyde dehydrogenase by NAD^+ to acetic acid:

$$CH_3CHO + NAD^+ + H_2O \xrightarrow{AldDH} CH_3COOH + NADH + H^+$$

The amount of NADH formed was stoichiometric with the amount of acetaldehyde present, and the concentration of NADH was determined by means of its absorbance at 340 nm.

MEASUREMENT OF FREE SULPHITE CONCENTRATIONS.

The method described by Burroughs and Sparks (1964) was used. A sample (5 ml) was placed in a 250 ml round two-necked flask. Orthophosphoric acid (5 ml; 25%, v/v) was added and the flask connected from one neck to a series of two boiling tubes by polyvinyl-chloride piping and cone joints fitted with 12 cm delivery tubes. The pressure in the apparatus was lowered to 80 mm Hg using a water pump whilst a steady stream of air was bubbled through the flask contents carrying sulphur dioxide to the boiling tubes which contained a trapping agent. The trapping agent consisted of 90 ml water supplemented with 3.3 ml 100-volume hydrogen peroxide and 1 ml Tashiro indicator (equal volumes of 0.2% (w/v) methyl red and 0.1% (w/v) methylene blue, both in 95% (v/v) ethanol). The mixture was neutralized with sodium hydroxide (10 mM) to a grey colour and made up to 100 ml with water. After 15 min, the vacuum was shut off and the contents of the first tube were titrated with sodium hydroxide (1 mM) from a red colour to a grey end-point. A further tube containing 5 ml trapping agent was attached to the apparatus and the experiment resumed for 15 min; titration of the contents of this tube gave a reagent-blank value which was subtracted from the first titre. The free sulphur dioxide equivalent was determined from the relationship:

$$0.032 \text{ mg } SO_2 \equiv 1 \text{ ml NaOH (1 mM)}$$

Any sulphur dioxide not retained in the first tube was readily detected by a colour change in the second.

ADDITION OF ACID TO YEAST CELLS.

Harvested organisms were washed twice in ice-cold distilled water and resuspended to approx. 20 mg dry wt. ml^{-1} in distilled water or 50 mM glucose solution. Cell suspension density was adjusted by dilution to 10 mg dry wt. ml^{-1} and portions (10 ml) were titrated against hydrochloric acid (50 mM). Suspensions were maintained at 30°C and stirred magnetically. Additions of acid were made at pre-determined time intervals, and the pH value of the suspension was continuously monitored (Pye Unicam PW 9418 pH meter). In certain experiments, cell suspensions were, prior to acid addition, killed by boiling on a water bath for 5 min.

MEASUREMENT OF BUFFERING CAPACITY OF YEAST CELL FRACTIONS.

(a) MEASUREMENT OF CELL-WALL BUFFERING CAPACITY.

(i) Disruption of Cells by Homogenization and Preparation of Isolated Cell Walls.

Harvested organisms were washed twice in ice-cold distilled water and the cell washings retained at -20°C. Organisms were resuspended to approx. 20 mg dry wt. ml^{-1} in ice-cold 50 mM Tris-HCl (pH 8.5) buffer containing phenylmethylsulphonyl fluoride (PMSF; 1 mM) and added to a 50 ml Braun bottle (FT Scientific Instruments Ltd., Bredon, Tewkesbury, England) containing 30 g

glass beads (0.45 - 0.50 mm diameter). The bottle was stoppered and shaken in a Braun oscillator for six periods of 15 s at 4000 rev. min^{-1} with constant cooling from expansion of compressed carbon dioxide. Using Tris-HCl buffer (5 - 20 ml) to rinse out the bottle contents, the suspension was transferred to a 40 ml centrifuge tube. Following centrifugation (1300 *g*; 15 min; 4°C), the supernatant was pipetted off and retained on ice. Cell walls and unbroken cells were separated from glass beads by suspending in ice-cold buffer and allowing the beads to settle out by keeping the suspension on ice for 1 min; the supernatant was combined with that retained previously. Combined supernatant was centrifuged as already described and the pellet (consisting of whole cells and cell walls) was washed five times in ice-cold buffer, each time transferring to a glass centrifuge tube the white cell-wall layer which lay on top of the yellow cell-pellet. In certain experiments, plasma-membrane fractions were removed by twice suspending cell-wall material in ethanol (5 ml) for 10 min, and repeating the process two more times using chloroform : methanol (1:1) and ethanol : diethyl ether (1:1) mixtures, respectively. Cell walls were finally washed ten times in ice-cold distilled water and retained at -20°C. Isolated cell walls were then freeze-dried. Cell wall purity was assessed directly by light microscopy and also by the following staining method. A thin film of cell-wall material was heat-fixed onto a microscope slide, covered with Sudan Black B (0.3%, w/v in 70%, v/v ethanol) and held at room temperature for 15 min. Stain was drained off and the slide blotted dry. The smear was then flushed with xylene, dried, and a counter-stain of safranin applied for 5 - 10 s before rinsing with water. Under the light microscope lipid-free material appeared red, whereas lipid-containing material appeared predominantly black.

(ii) Titration of Cell Walls Against Acid.

Freeze-dried cell wall material was suspended to 0.1% (w/v) in distilled water and titrated against hydrochloric acid (5 - 250 mM) whilst monitoring the pH

value of the suspension. Cell washings retained from yeast harvesting were titrated against acid in the same manner.

(b) MEASUREMENT OF CELL-CYTOSOL BUFFERING CAPACITY.

(i) Measurement of Yeast DNA Content.

A technique which was based on the method of Burton (1955) and described by Stewart (1975) to assay perchloric acid-extracts of cells with diphenylamine-based reagent was used. Harvested organisms were washed twice in ice-cold distilled water and resuspended to approx. 20 mg dry wt. ml^{-1} in water. A portion (1 ml) of perchloric acid (0.5 M) was added to cell suspension (1 - 2 ml) in a 10 ml centrifuge tube and incubated on ice for 15 min with periodic shaking to keep the cells in suspension. Following centrifugation (5000 *g*; 5 min) the supernatant was discarded and the pellet resuspended in a mixture of ice-cold water (1 ml) and 0.5 M perchloric acid (1 ml). The suspension was incubated on ice as already described and the cells recovered by centrifugation. Cells were then resuspended in 0.5 M perchloric acid (1 ml) and heated at 70°C for 15 min. The heated suspension was centrifuged, the supernatant retained and the extraction at 70°C repeated two more times. Supernatants were pooled and the extract volumes made up to 5 ml with 0.5 M perchloric acid for assay with diphenylamine. Diphenylamine reagent consisted of 1.5% (w/v) diphenylamine dissolved in concentrated glacial acetic acid containing 1.5% (v/v) sulphuric acid. Immediately prior to use, a portion (0.5 ml) of acetaldehyde solution (16 mg ml^{-1}) was added to each 100 ml of reagent. A portion (2 ml) of sample extract was mixed with an equal volume of diphenylamine reagent and incubated at 30°C for 20 h. The absorbance of the solutions was measured at 600 nm against a reagent blank and compared with standards containing up to 100 μg DNA. A standard curve was produced by assaying salmon-testes DNA. Equal volumes of DNA stock solution (200 μg DNA ml^{-1} in 5 mM sodium

hydroxide) and perchloric acid (1 M) were mixed and incubated for 15 min before adding diphenylamine reagent in parallel with the sample extracts.

(ii) Disruption of Yeasts Using Ultrasound.

Organisms were disrupted by ultrasonic cavitation in which the physical effects of the collapse of sound-generated bubbles break yeast cells in suspension (Lloyd and Coakley, 1979). Portions (up to 40 ml) of cell suspension (approx. 20 mg dry wt. ml^{-1}) were transferred to a sonication vessel. A probe (2 cm diameter) was immersed approx. 1 mm below the surface of the suspension which was then sonicated at a frequency of 20 kHz. Optimum disruption times were ascertained from standard curves relating absorbance at 600 nm and sonicating time for each yeast strain. During sonication the suspension was continuously cooled by circulation of water around the jacket of the sonication vessel which was immersed in ice so that the temperature of the suspension did not exceed 5°C.

(iii) Titration of Cell Cytosols Against Acid.

Harvested organisms were washed twice in ice-cold distilled water and resuspended to approx. 20 mg dry wt. ml^{-1}. Portions of suspension (4 x 1 ml) were removed for measurement of DNA content, and a further 2 x 0.5 ml samples were removed for measurement of absorbance and related to dry wt. value by standard curves prepared for each yeast strain. The remaining suspension (approx. 40 ml) was sonicated, as already described. Sonicated suspension was centrifuged (1300 *g*; 15 min; 4°C) and the supernatant re-centrifuged under the same conditions; the volume of second supernatant (cell cytosols) was recorded. Pellets from both centrifugation procedures were retained and combined. Portions (10 ml) of cell cytosols were transferred to 25 ml beakers maintained on ice, and titrated against

20 mM hydrochloric acid whilst monitoring the pH value of the cytosols. The volume of hydrochloric acid required to lower the pH value of the cell cytosols by one unit was recorded. The combined pellet was washed three times in 10 mM Tris-HCl (pH 7.0) buffer (4°C) and resuspended in a volume of buffer equal to the volume of cell cytosols recorded before. Portions of suspension (4 x 2 ml) were removed for measurement of DNA content. The quantity of cells disrupted by ultrasound was calculated from the initial yeast dry wt. value and equivalent DNA content, and the DNA content of intact cells after sonication. The buffering capacity refers to the ability of a solution to resist pH change and is defined as the number of moles of acid or base that must be added to each litre of solution to produce a unit change in pH.

MEASUREMENT OF SULPHITE ACCUMULATION.

Harvested organisms were washed twice in 67 mM citric acid / 77 mM dipotassium hydrogen orthophosphate (pH 4.0) buffer containing 100 mM glucose and resuspended to 10 mg dry wt. ml^{-1} in the same buffer. Cell suspension was maintained at 30°C in a constant-temperature water bath and stirred magnetically. After 3 min equilibration, a portion of suspension was transferred to a reaction mixture which consisted of citric acid buffer, glucose (100 mM) and 1 mg dry wt. organisms ml^{-1}. The reaction mixture was held for a further 2 min and the measurement started by adding a portion (0.5 - 1.0 ml) of sodium metabisulphite solution containing sodium [^{35}S]sulphite to give final concentrations of sulphite ranging between 50 μM and 8 mM (0.2 - 0.6 μCi ml^{-1}; 1 μCi = 37 kBq). At appropriate time intervals, 1 ml portions were taken from suspension and rapidly passed through membrane filters (0.45 μm pore size; 25 mm diameter) which had been pre-washed with citric acid buffer containing non-radioactive sulphite at the concentration used in the experiment; immediately after filtration organisms on

filters were washed with the same ice-cold buffer (4 x 1 ml). Once the time for equilibration of sulphite had been ascertained, replicate measurements were obtained by sampling at that time. The absorbance of the suspension was measured to determine the equivalent dry wt. value of the mixture by relating to standard curves prepared for each yeast strain. Filters with organisms were placed in scintillation vials containing 7 ml Optiphase Safe scintillation fluid (Fisons Laboratory Supplies, Loughborough, Leicestershire, England). Radioactivity in the vials was measured in a LKB Liquid Scintillation Counter (model 1217; LKB Instruments Ltd., South Croydon, Surrey, England). Background activity was measured by repeating the procedure without organisms to make sure that sulphite was not binding to filters. Sodium [^{35}S]sulphite was stored in 0.5 ml portions (0.2 mCi ml^{-1}) at -20°C in 5 mM EDTA under nitrogen gas to prevent oxidation.

MEASUREMENT OF INTRACELLULAR WATER VOLUME.

The method used was based on the procedure of Cala (1977). Harvested cells were washed twice in 67 mM citric acid / 77 mM dipotassium hydrogen orthophosphate (pH 4.0) buffer containing 100 mM glucose and resuspended to approx. 12.5 mg dry wt. ml^{-1} in the same buffer. Organisms were then suspended to 5 mg dry wt. ml^{-1} in citric acid buffer containing glucose (100 mM) and [1-^{14}C]mannitol (10 mM; 0.024 μCi ml^{-1}) in a 50 ml beaker and maintained at 4°C with stirring. The absorbance of the suspension was measured to determine the equivalent dry wt. value of the mixture by relating to standard curves prepared for each yeast strain. After a 10 min incubation period, duplicate 0.2 ml samples were removed and passed through membrane filters (0.45 μm pore size; 25 mm diameter) which had been washed with citric acid buffer containing non-radioactive mannitol (10 mM); immediately after filtration organisms on filters were washed with the same buffer (4 x 1 ml). Filters with organisms were transferred to scintillation vials

containing 7 ml scintillation fluid and radioactivity measured using a LKB Liquid Scintillation Counter (model 1217). Background activity was measured by repeating the procedure without organisms to make sure that mannitol was not binding to filters. These measurements established that mannitol was not taken into the cells. In addition, aliquots (12 x 1.4 ml) of cell suspension were centrifuged for 5 min at 11 500 *g* in pre-dried Eppendorf tubes. Portions (0.2 ml) of supernatant were transferred from each tube to scintillation vials for measurement of radioactivity, as already described. The remaining supernatant was discarded and the last traces removed from the pellet surface with tissue paper. After establishing the wet weight of cell pellets, Eppendorf tubes were dried in a vacuum oven at 55°C to constant weight. The tubes were re-weighed to establish the amount of water lost from each pellet. Dried pellets were rehydrated with 0.2 ml distilled water and transferred to scintillation vials for measurement of radioactivity.

MEASUREMENT OF PROPIONIC ACID ACCUMULATION AND CALCULATION OF INTRACELLULAR pH VALUE.

Intracellular pH values of organisms were calculated by determining the equilibrium distribution of propionic acid across the plasma membrane (Conway and Downey, 1950). Harvested organisms were washed twice with 67 mM citric acid / 77 mM dipotassium hydrogen orthophosphate (pH 4.0) buffer containing 100 mM glucose and resuspended to approx. 10 mg dry wt. ml^{-1} in the same buffer. Cell suspension was maintained at 30°C in a constant-temperature water bath and stirred magnetically. After 3 min equilibration, a portion of suspension was transferred to produce a reaction mixture which contained citric acid buffer, glucose (100 mM) and 1 mg dry wt. organisms ml^{-1}. The reaction mixture was held for a further 2 min and the measurement was started by adding a portion (0.5 - 1.0 ml) of propionic acid solution containing sodium [2-^{14}C]propionate to give a final

concentration of propionic acid of 2μM (0.5 μCi ml^{-1}). At appropriate time intervals, duplicate 1 ml portions were taken from suspension and rapidly passed through membrane filters (0.45 μm pore size; 25 mm diameter) which had been pre-washed with citric acid buffer containing non-radioactive propionic acid (2 μM); immediately after filtration organisms on filters were washed with the same ice-cold buffer (4 x 1 ml). Once the time for equilibration of propionic acid had been ascertained, replicate measurements were obtained by sampling at that time. The absorbance of the suspension was measured to determine the equivalent dry wt. value of the mixture by relating to standard curves prepared for each yeast strain. Filters with organisms were transferred to scintillation vials containing 7 ml scintillation fluid and radioactivity measured using a LKB Liquid Scintillation Counter (model 1217). Background activity was measured by repeating the procedure without organisms to make sure that propionic acid was not binding to filters. Intracellular pH values were calculated from the expression

$$pHi = pKi + \log_{10} \left[\frac{TAiVe}{TAeVi} \left(10^{(pHe-pKe)} + 1\right)-1 \right]$$

(Waddell and Butler, 1959). The variables pHi and pHe are the intracellular and extracellular pH values, TAi and TAe are the intracellular and extracellular total amounts of propionic acid and Vi and Ve are the intracellular and extracellular volumes. Dissociation constants for propionic acid in the internal and external environments, pKi and pKe, were calculated to be 4.75 and 4.86, respectively (Pilkington and Rose, 1988).

CHEMICALS

All chemicals used were of AnalaR grade or of the highest purity available. All radioactively-labelled compounds were obtained from Amersham International plc, Aylesbury, Buckinghamshire, England.

MATERIALS AND METHODS (PART B)

ORGANISM.

The yeast used to pitch fermentations was *Saccharomyces cerevisiae* AWRI 1A65 which was maintained as described in Materials and Methods (Part A).

EXPERIMENTAL FERMENTATIONS.

Unless otherwise stated, fermentations were carried out using apple-juice medium which consisted of (l^{-1}) apple-juice concentrate (84 ml), glucose syrup (118 ml; 160 g), ammonium sulphate (0.1 g), ammonium carbonate (0.1 g), thiamin solution (0.01 %, w/v; 0.22 ml) and distilled water (798 ml). Apple juice concentrate and glucose syrup were kindly supplied by HP Bulmer Ltd., and were stored at 4°C. Starter cultures consisting of 150 ml sterile (121°C; 1 min) apple-juice medium in 250 ml conical flasks were inoculated with a pinhead of yeast and incubated for four days at 20°C on an agitating water bath. Fermentations were carried out in two-litre round, flat-bottomed flasks each with two necks, one of which was fitted with a Suba-seal for sampling and the other a fermentation lock. Portions of potassium metabisulphite solution (250 mM) were added to apple-juice medium to produce final concentrations of sulphite ranging between zero and 3.12 mM, and the medium was held at 20°C for a 20 h equilibration period prior to pitching. One-litre portions of apple-juice medium were inoculated with a volume of starter culture to produce a concentration of 5.0×10^6 cells ml^{-1} as determined using an Improved Neubauer counting chamber. Fermentations were maintained at 20°C and stirred magnetically (4 cm stirring bar; approx. 200 rev. min^{-1}). Anaerobic conditions in each fermentation were self-induced. In certain

experiments, apple-juice medium was inoculated with wild yeast isolates before pitching, to produce a population equivalent to that found in fresh apple juice. Wild yeasts were isolated from fresh apple juice obtained from HP Bulmer Ltd. during autumn 1989; serial dilutions of the juice were prepared and plated onto Wallerstein Laboratories Nutrient (W.L.N.) medium. Plates were incubated for 5 - 7 days at 20°C and the colonies enumerated. Each morphologically distinct yeast was inoculated onto a slope of M.Y.G.P. medium and incubated for 3 - 5 days at 20°C; slopes were then stored at 4°C. Wild yeasts were propagated by inoculating 50 ml portions of sterile concentrate-derived apple juice and incubating at 20°C, with shaking, for three days. After this period, yeasts were counted using an Improved Neubauer counting chamber, and added in appropriate numbers to one-litre portions of concentrate-derived apple juice.

DETECTION OF WILD AND PITCHING YEAST POPULATIONS.

Viable cells were estimated by serial dilution and plating of dilutions onto W.L.N. medium, Lysine medium containing bromocresol green (0.002%, w/v), and sulphite-containing medium. Sulphite-containing medium consisted of (l^{-1}) glucose (60 g), yeast extract (Lab M; 5 g), agar (Lab M, No. 2; 35 g), and was buffered to pH 4.0 by addition of citric acid (5.7 g) and sodium citrate (6.0 g). Following autoclaving (121°C; 15 min), potassium metabisulphite (2.6 g) was added. Plates were incubated at 20°C for one week (W.L.N. and Lysine media) or ten days (sulphite-containing medium) after which time the number of colonies on each plate was recorded.

MEASUREMENT OF ETHANOL CONCENTRATIONS.

Portions of culture were centrifuged and the supernatant retained. A portion of supernatant was diluted as necessary with water and mixed with an equal volume of 0.2% (v/v) butan-1-ol in water. A sample (1 μl) was injected into the column of a Pye Unicam PU4500 gas chromatograph fitted with an inlet split (set at a ratio of 1:60 injected : discarded) and a flame ionization detector. The column (25AQ2 BP20-0.25; SGE Ltd.) was a capillary 25 m in length and of 0.22 mm internal diameter, with a film thickness of 0.25 μm, and was clad in aluminium. The column was maintained at 140°C. The injection and detector temperatures were 200°C and 150°C, respectively. The column carrier gas (helium) flow rate was 1 ml min^{-1} and nitrogen gas (40ml min^{-1}) was passed through the detector. Ethanol concentrations were calculated by comparing areas of the ethanol peaks with those of the butan-1-ol peaks. Response factors were calculated which compared butan-1-ol and ethanol in equal concentrations.

CHEMICALS.

All chemicals used were of AnalaR grade or of the highest possible purity. Wallerstein Laboratories Nutrient and Lysine media were obtained from Unipath Ltd., Basingstoke, Hampshire, England. Gas chromatography columns were obtained from Scientific Glass Engineering Pty. Ltd., Kiln Farm, Milton Keynes, England.

RESULTS.

RESULTS (PART A)

GROWTH OF ORGANISMS.

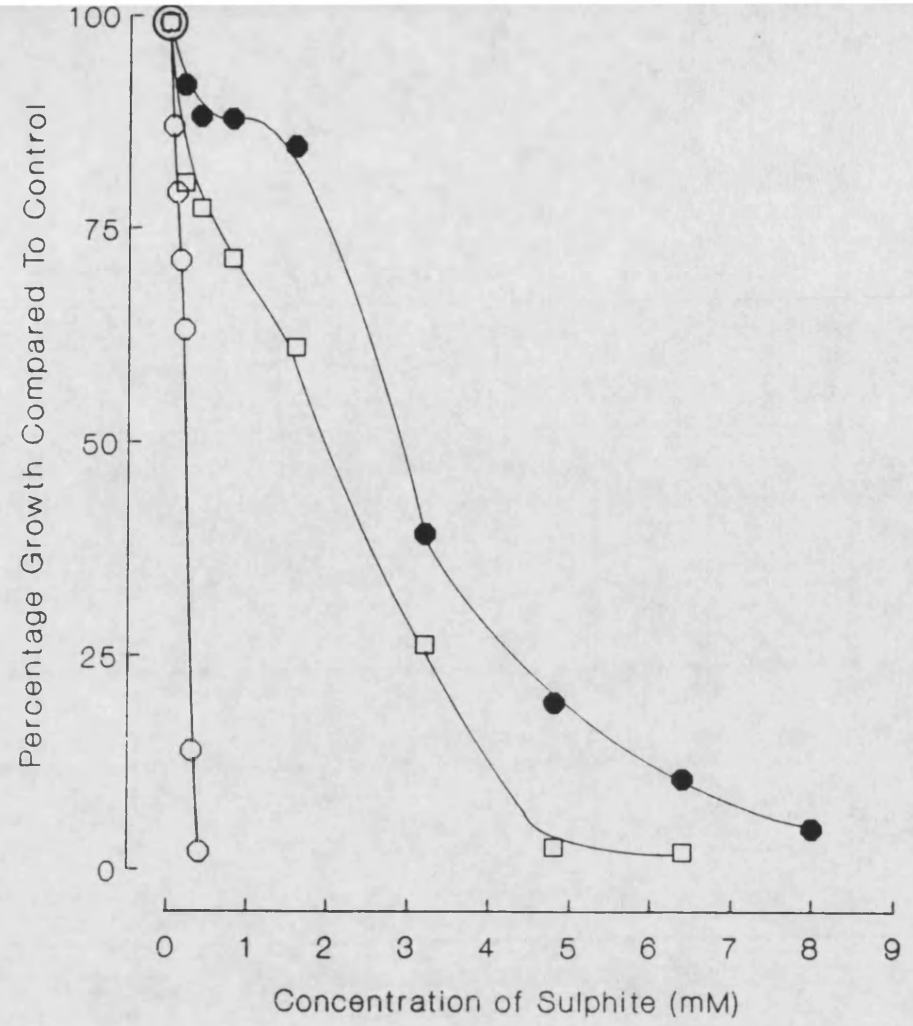
Organisms grown aerobically reached mid-exponential phase after approx. 23 h for *Sacch. cerevisiae* AWRI 1A65, 22 h 30 min for *S'codes ludwigii* BC1 and 21 h for *S'codes ludwigii* TC10. The generation time during exponential growth for *Sacch. cerevisiae* AWRI 1A65 was 3 h, for *S'codes ludwigii* BC1 2 h and for *S'codes ludwigii* TC10 2 h 30 min. Final growth yields in the stationary phase were approx. 1.3 mg dry wt. ml^{-1} for *Sacch. cerevisiae* AWRI 1A65 and *S'codes ludwigii* TC10, and 1.2 mg dry wt. ml^{-1} for *S'codes ludwigii* BC1. Plots of absorbance (600 nm) against (mg dry wt. organisms) ml^{-1} were all found to be linear up to at least A_{600nm} 0.6. Values of cell volume were 1.179 $\pm$ 0.076, 1.725 $\pm$ 0.234 and 2.291 $\pm$ 0.102 μl (mg dry wt.)$^{-1}$, respectively, for *Sacch. cerevisiae* AWRI 1A65, *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10 at early- and mid-exponential phases; at stationary phase, cell volumes were, respectively, 1.045 $\pm$ 0.066, 1.661 $\pm$ 0.202 and 2.128 $\pm$ 0.113 μl (mg dry wt.)$^{-1}$. In keeping with the findings of Pilkington (1988), cell volumes were found to remain constant in the presence of sulphite.

EFFECT OF SULPHITE ON YEAST GROWTH AND VIABILITY.

Sulphite inhibited growth, to different extents, of all three strains, as measured using microtitre plates (Fig. 1). *Saccharomyces cerevisiae* AWRI 1A65 was most sensitive to sulphite, growth of this organism being completely inhibited by 0.4 mM total sulphite. *Saccharomycodes ludwigii* BC1 was least sensitive, and was able to grow at sulphite concentrations which prevented growth of *S'codes ludwigii* TC10.

Figure 1. Effect of sulphite concentration on growth of *Saccharomyces cerevisiae* AWRI 1A65 (Figs. 1a and b; ○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□) in medium in microtitre wells. Values given are the means of measurements on at least eight separate plates. The maximum variation was ± 7.3%.

Figure 1a


100
75
50
25
0
Percentage Growth Compared To Control
0 1 2 3 4 5 6 7 8 9
Concentration of Sulphite (mM)

1b

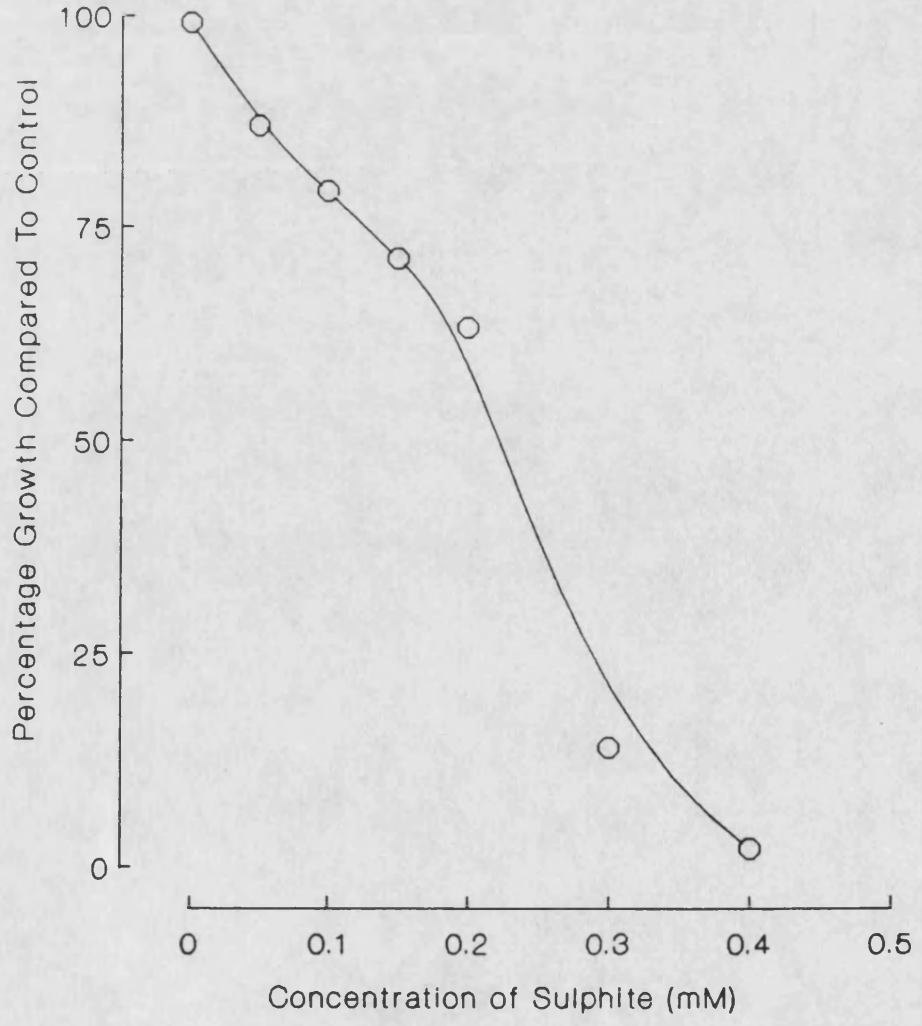
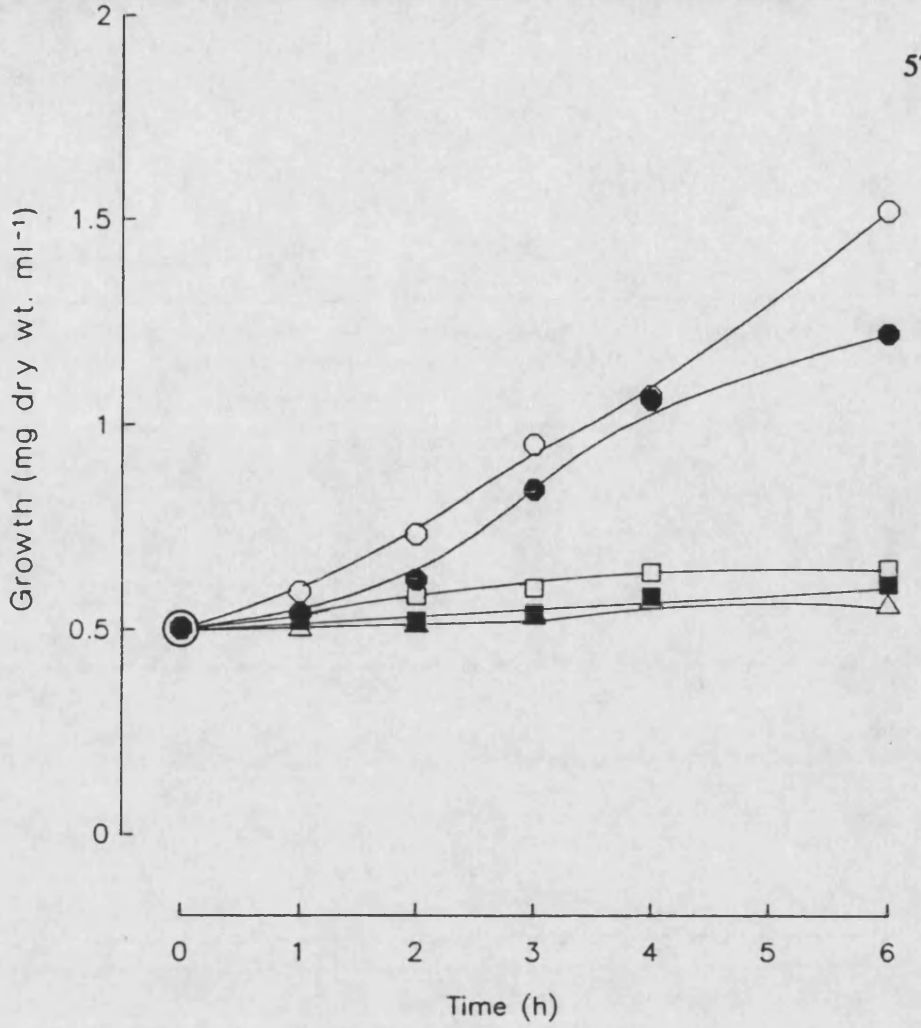

100
75
50
25
0
Percentage Growth Compared To Control
0 0.1 0.2 0.3 0.4 0.5
Concentration of Sulphite (mM)

Figure 2. Time-course of (i) growth and (ii) change in viability of *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 2a), *Saccharomycodes ludwigii* BC1 (Fig. 2b) and *Saccharomycodes ludwigii* TC10 (Fig. 2c) incubated with stirring at 30°C in one-litre portions of media either lacking sulphite (○) or containing 0.16 mM (●), 0.39 mM (□), 0.78 mM (■), 1.56 mM (△), 3.12 mM (▲) or 7.81 mM (◇) total sulphite. Values given are the means of duplicate determinations. The maximum variation in values for growth (mg dry wt. ml^{-1}) was ± 0.05, and for viability (log_{10} cfu ml^{-1}) maximum variation was ± 0.40.

Figure 2a(i)



2a(ii)

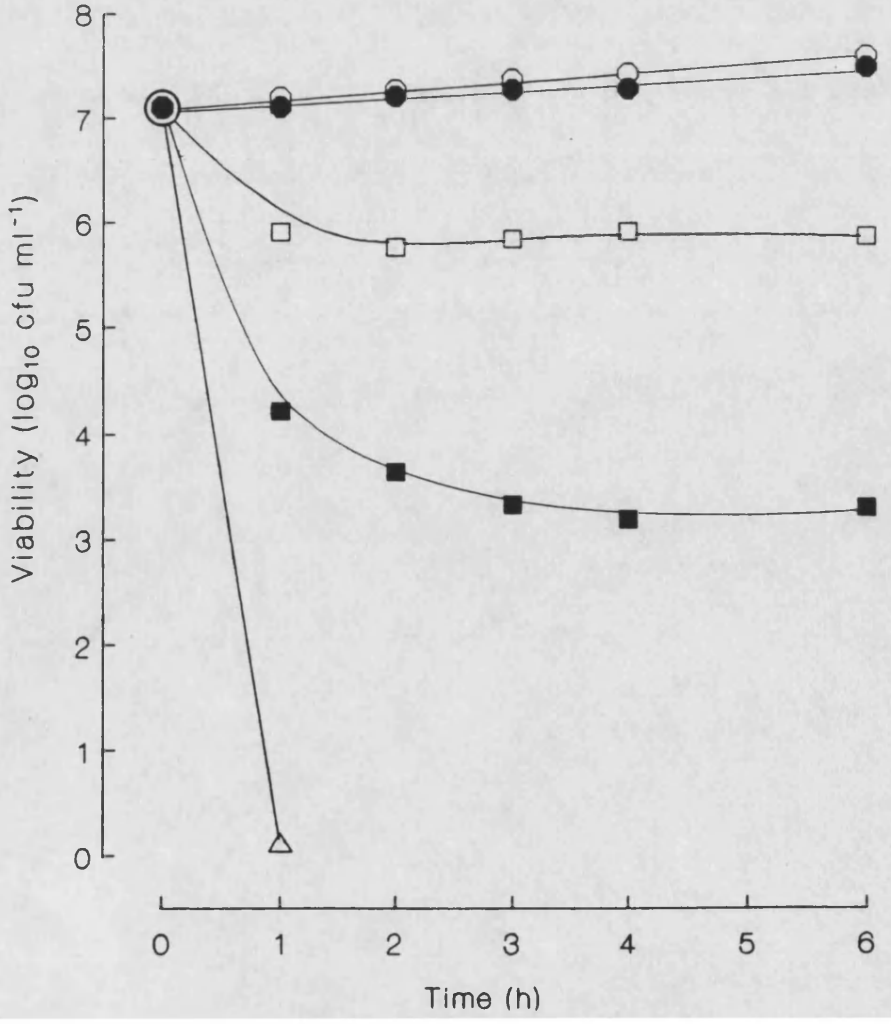
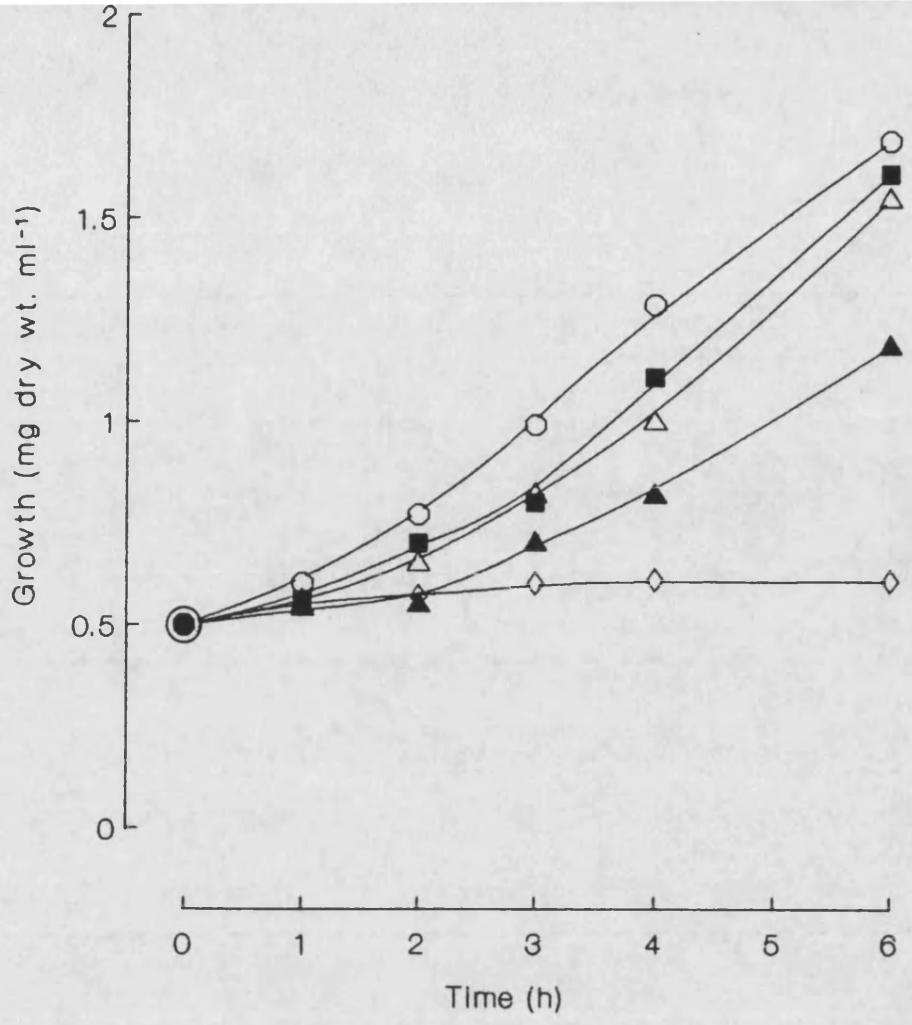


Figure 2b(i)



2b(ii)

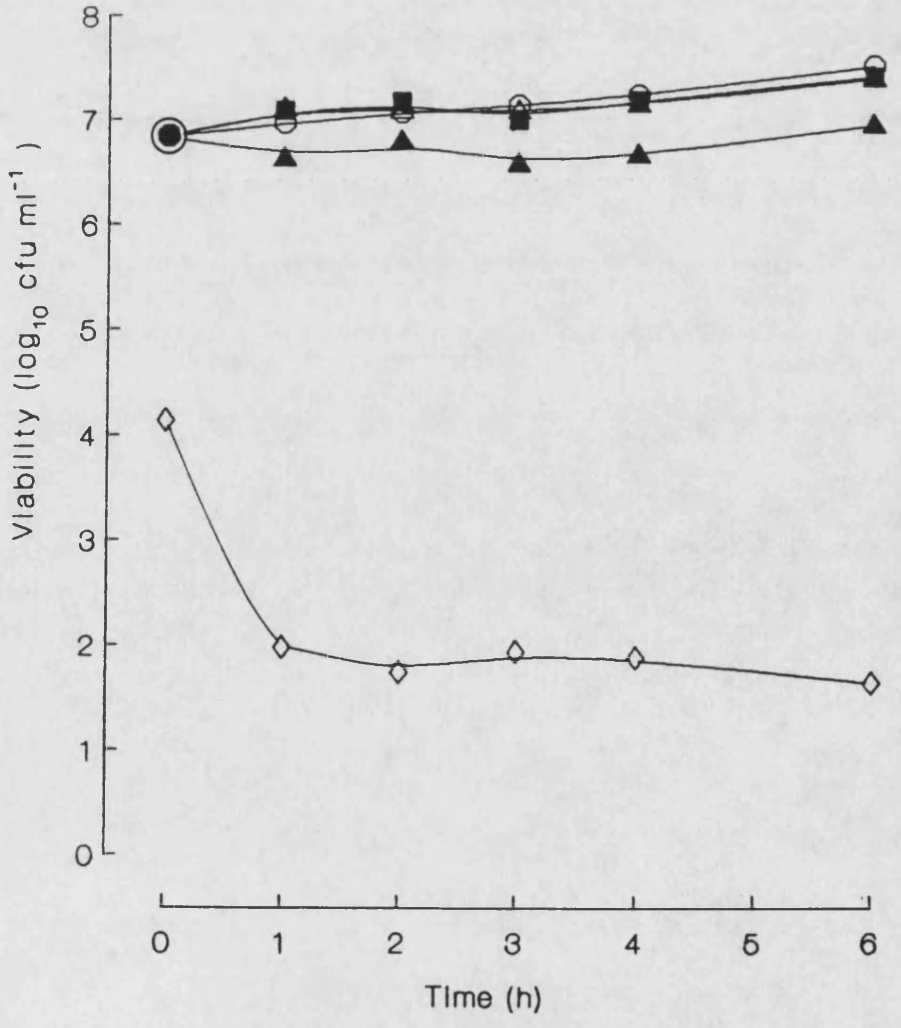
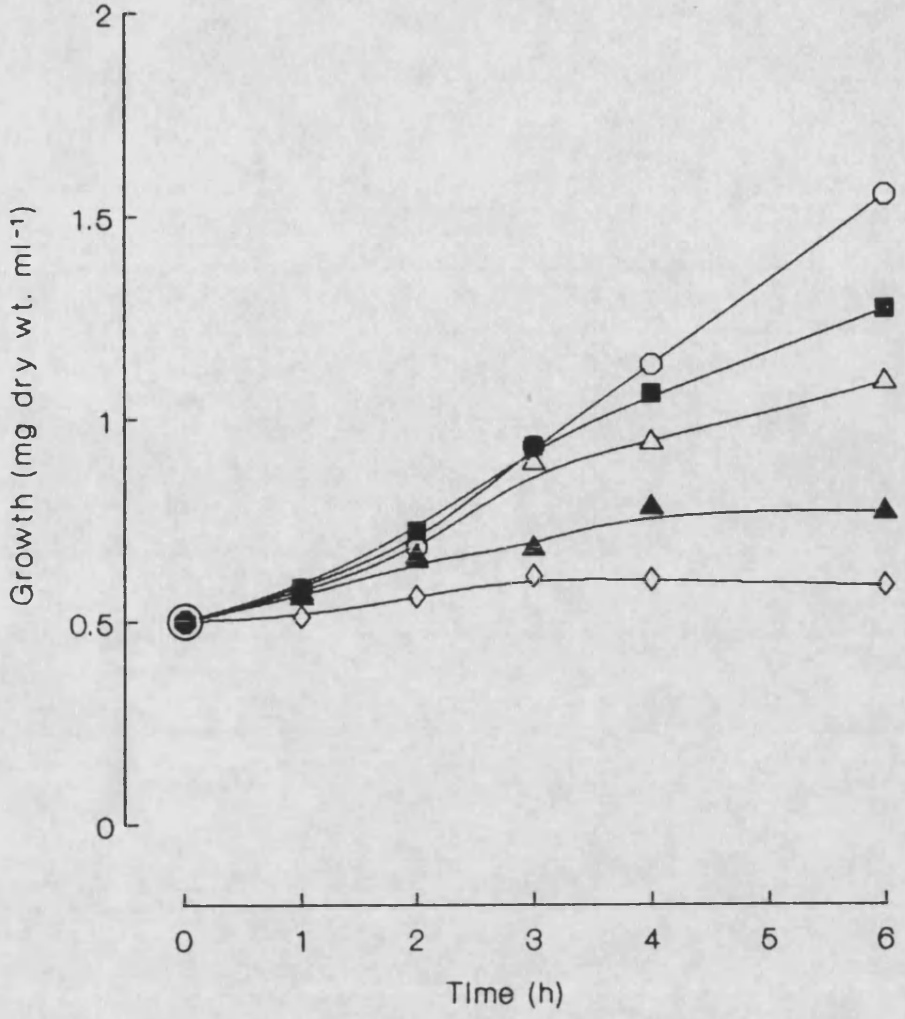
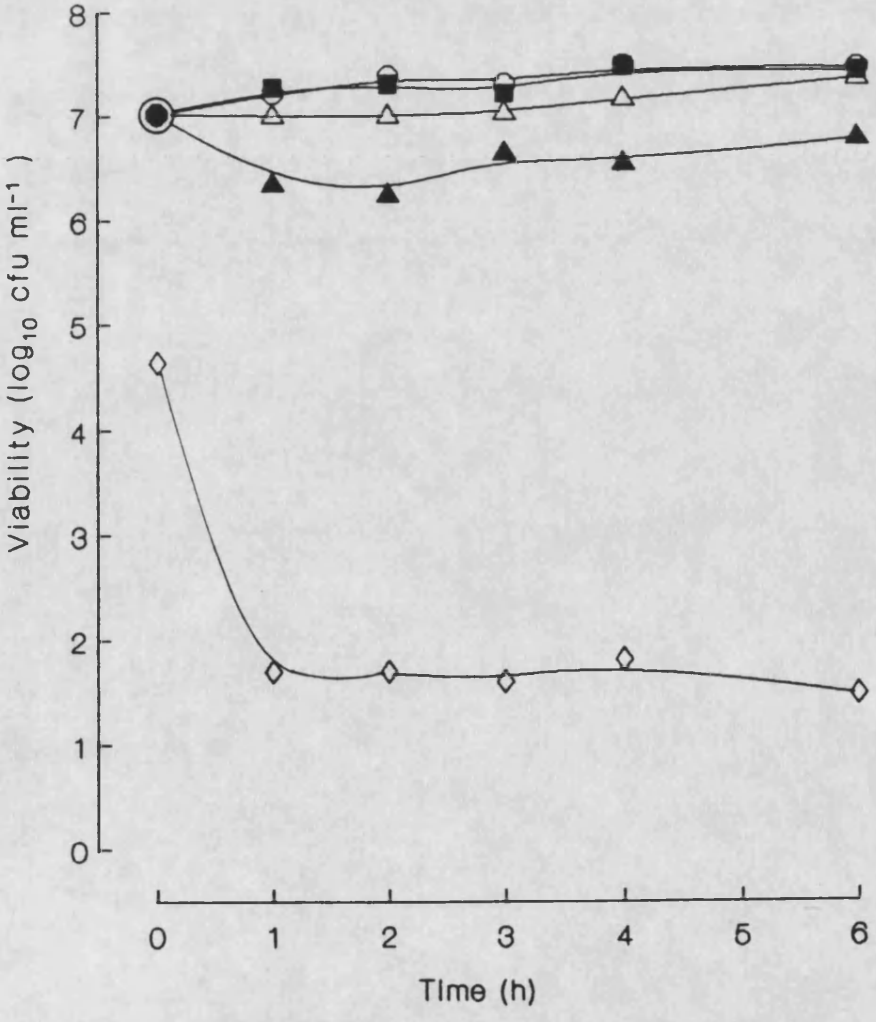


Figure 2c(i)



2c(ii)



Total Sulphite Added (mM)	Free Sulphite Present (mM)
0.16	0.12 ± 0.01
0.39	0.25 ± 0.01
0.78	0.62 ± 0.02
1.56	1.38 ± 0.04
3.12	2.78 ± 0.02
7.81	6.86 ± 0.08

Table 1. Total sulphite added to one-litre portions of media and free sulphur dioxide concentrations remaining after 10 min, as determined using the method of Burroughs and Sparks (1964). Values quoted for free sulphite are the means of at least three independent determinations ± S.D.

In one-litre portions of medium, *Sacch. cerevisiae* AWRI 1A65 demonstrated greatest sensitivity to sulphite, and *S'codes ludwigii* BC1 least sensitivity (Fig. 2). Growth of *Sacch. cerevisiae* AWRI 1A65 was impaired by 0.16 mM and almost completely inhibited by 0.39 mM total sulphite. Viability of *Sacch. cerevisiae* AWRI 1A65 was not significantly decreased at 0.16 mM total sulphite but, at concentrations of 0.39 mM and above, viability declined. Cultures of *Sacch. cerevisiae* AWRI 1A65 were killed by 1.56 mM total sulphite. Growth of *S'codes ludwigii* BC1 was increasingly impaired at concentrations above 0.78 mM, and was completely inhibited by 7.81 mM total sulphite. Viability of *S'codes ludwigii* BC1 was decreased by a concentration of 3.12 mM total sulphite. At a concentration 7.81 mM total sulphite, a proportion of the yeast population remained viable. Growth of *S'codes ludwigii* TC10 was impaired to a slightly greater extent than that of *S'codes ludwigii* BC1 at concentrations of 0.78, 1.56 and 3.12 mM total sulphite. In addition, viability of *S'codes ludwigii* TC10 was decreased to a greater extent than that of *S'codes ludwigii* BC1 at 3.12 mM total sulphite.

A sub-lethal concentration of sulphite was defined as one which impaired growth but did not decrease the viability of a yeast culture. On this basis, sub-lethal concentrations of total sulphite were designated as 0.16 mM for *Sacch. cerevisiae* AWRI 1A65 and 1.56 mM for strains of *S'codes ludwigii*. These concentrations corresponded to, respectively, 0.12 mM and 1.38 mM free sulphite initially present in the media (Table 1).

PRODUCTION OF ACETALDEHYDE BY YEASTS IN THE PRESENCE OF SULPHITE.

Acetaldehyde production was measured using organisms which were harvested in the mid-exponential phase of growth, and resuspended in one-litre

Figure 3. Time-course of the decline in the concentration of free sulphite (○) and of the increase in acetaldehyde concentration (●) in filtrates from cultures of *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 3a), *Saccharomycodes ludwigii* BC1 (Fig. 3b) and *Saccharomycodes ludwigii* TC10 (Fig. 3c) supplemented with sulphite at 0.16 mM for *Saccharomyces cerevisiae* AWRI 1A65 and 1.56 mM for both *Saccharomycodes* strains. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

Figure 3a

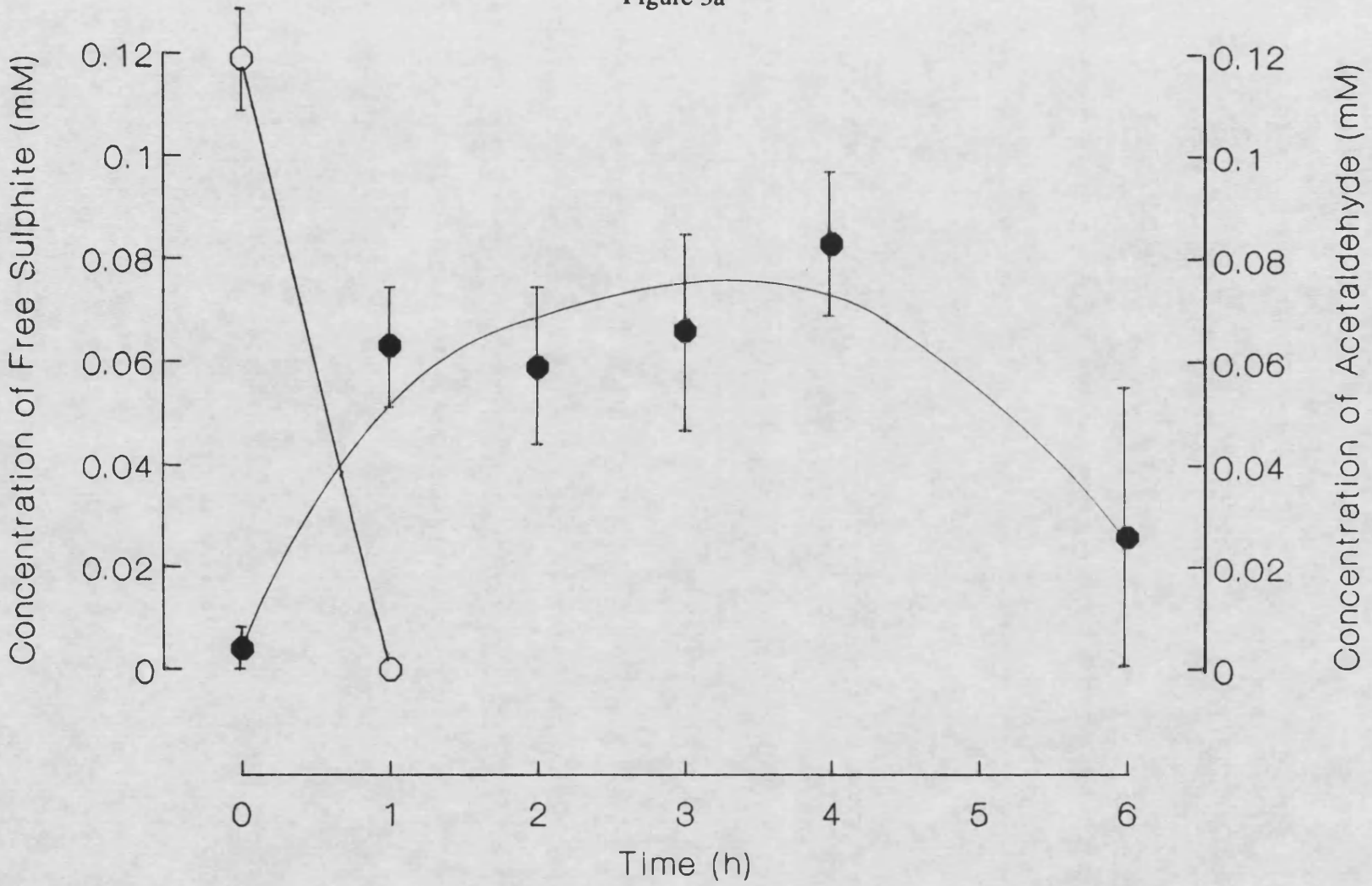
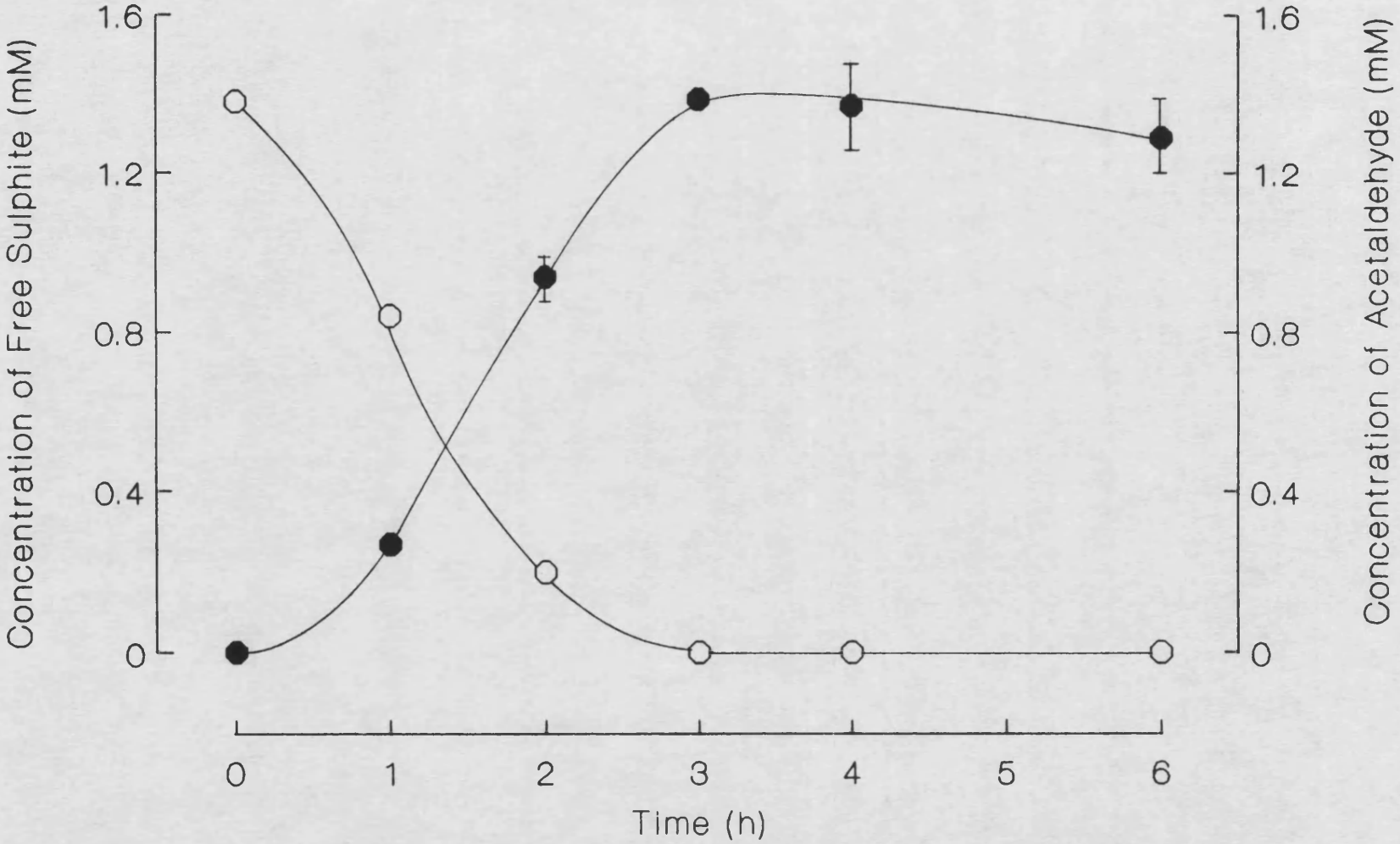

Concentration of Free Sulphite (mM)
0.12
0.1
0.08
0.06
0.04
0.02
0
Concentration of Acetaldehyde (mM)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
1
2
3
4
5
6
Time (h)

Figure 3b


Concentration of Free Sulphite (mM)
1.6
1.2
0.8
0.4
0
Concentration of Acetaldehyde (mM)
1.6
1.2
0.8
0.4
0
0
1
2
3
4
5
6
Time (h)

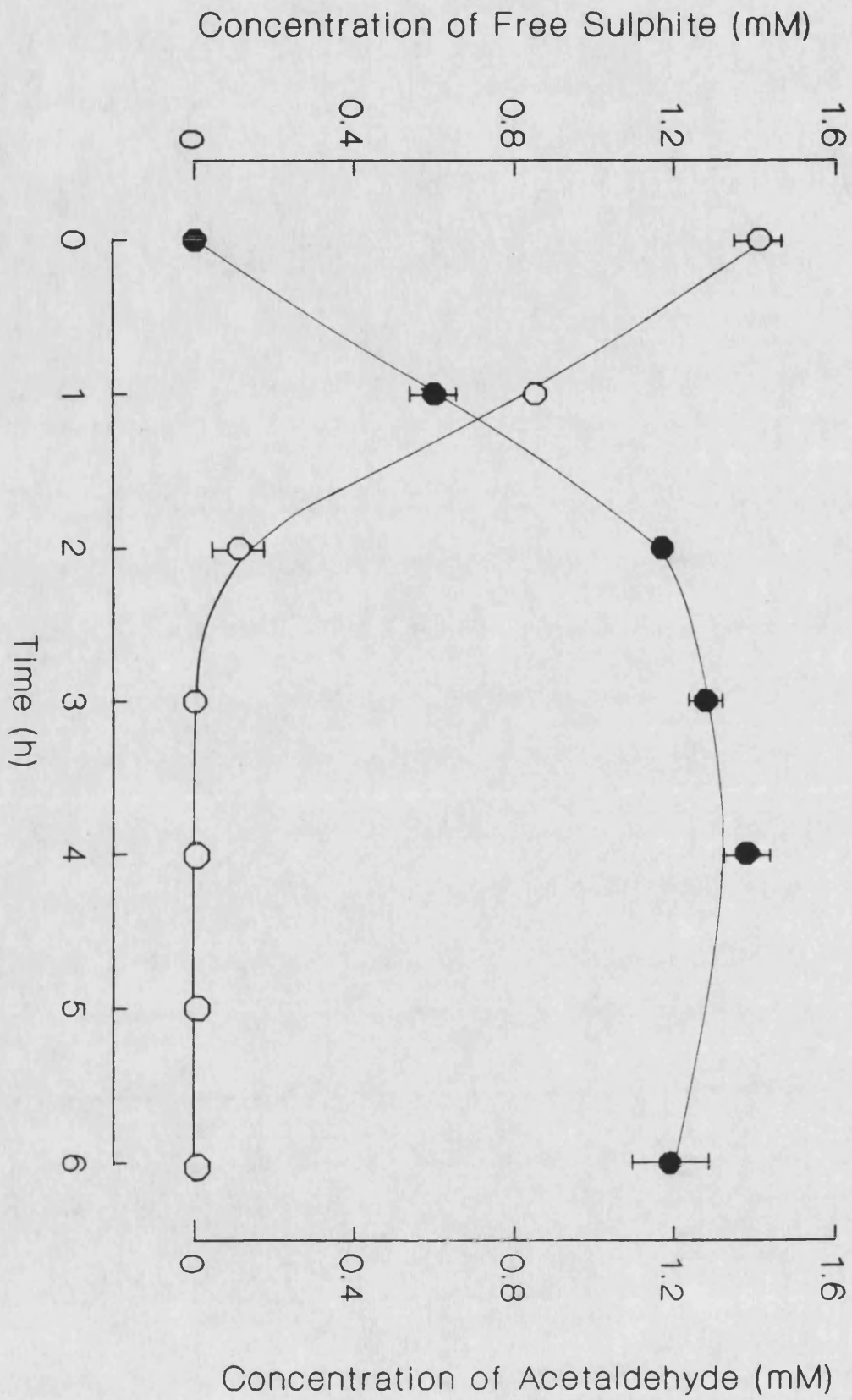

Concentration of Free Sulphite (mM)
0
0.4
0.8
1.2
1.6
Time (h)
0
1
2
3
4
5
6
Concentration of Acetaldehyde (mM)

Figure 3c

portions of fresh medium which had been supplemented with sub-lethal concentrations of sulphite. In this way, concentrations of free sulphite present in media immediately before yeast addition could be measured. *Saccharomyces cerevisiae* AWRI 1A65 produced a maximum concentration of 0.08 ± 0.02 mM acetaldehyde in media containing 0.12 ± 0.01 mM free sulphite (Fig. 3). *Saccharomycodes ludwigii* BC1 and *S'codes ludwigii* TC10 produced maximum concentrations of 1.39 ± 0.02 mM and 1.38 ± 0.06 mM acetaldehyde, respectively, in media containing 1.38 ± 0.02 mM and 1.41 ± 0.06 mM free sulphite. With all three yeasts, maximum concentrations of acetaldehyde were present after 3 - 4 h incubation. In cultures of both *S'codes ludwigii* strains, the increase in acetaldehyde concentrations closely mirrored the decrease in free sulphite concentrations. With cultures of *Sacch. cerevisiae* AWRI 1A65, however, all free sulphite became bound within one hour and maximum acetaldehyde concentrations were detected after 4 h incubation.

Ethanol concentrations of samples of filtrate were also measured. All three strains produced less ethanol in media supplemented with sulphite than in media which did not contain sulphite. After 6 h incubation in media, production of ethanol by *Sacch. cerevisiae* AWRI 1A65 amounted to 0.26 ± 0.03 mM in the absence of sulphite, and 0.16 ± 0.03 mM when a sub-lethal concentration of sulphite was present. Similarly, ethanol yields of *S'codes ludwigii* BC1 were reduced from 0.18 ± 0.02 to 0.15 ± 0.01 mM, and *S'codes ludwigii* TC10 from 0.25 ± 0.02 to 0.20 ± 0.02 mM, by sub-lethal concentrations of sulphite.

TITRATION OF YEAST SUSPENSIONS AGAINST ACID.

The time-course of titration of suspensions of organisms, which had been killed by boiling for 5 min, followed a simple 'staircase' character with all three strains (Fig. 4). Decreases in pH values of 0.6 - 0.7 unit were observed over the

Figure 4. Time-course of titration of yeast suspension lacking glucose (Y), suspension containing glucose (50 mM; Y+G) or suspension of boiled cells lacking glucose (BY) against 50 mM hydrochloric acid for *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 4a), *Saccharomycodes ludwigii* BC1 (Fig. 4b) and *Saccharomycodes ludwigii* TC10 (Fig. 4c). Suspensions (approx. 20 mg dry wt. ml^{-1}) were maintained at 30°C with stirring, and were not buffered. Arrows indicate times when portions of 50 mM hydrochloric acid were added to the suspension to increase the acid concentration in the suspension by 0.2 mM. Values given are the means of three independent determinations. Maximum S.D. for pH values was $\pm$ 0.10.

Figure 4a

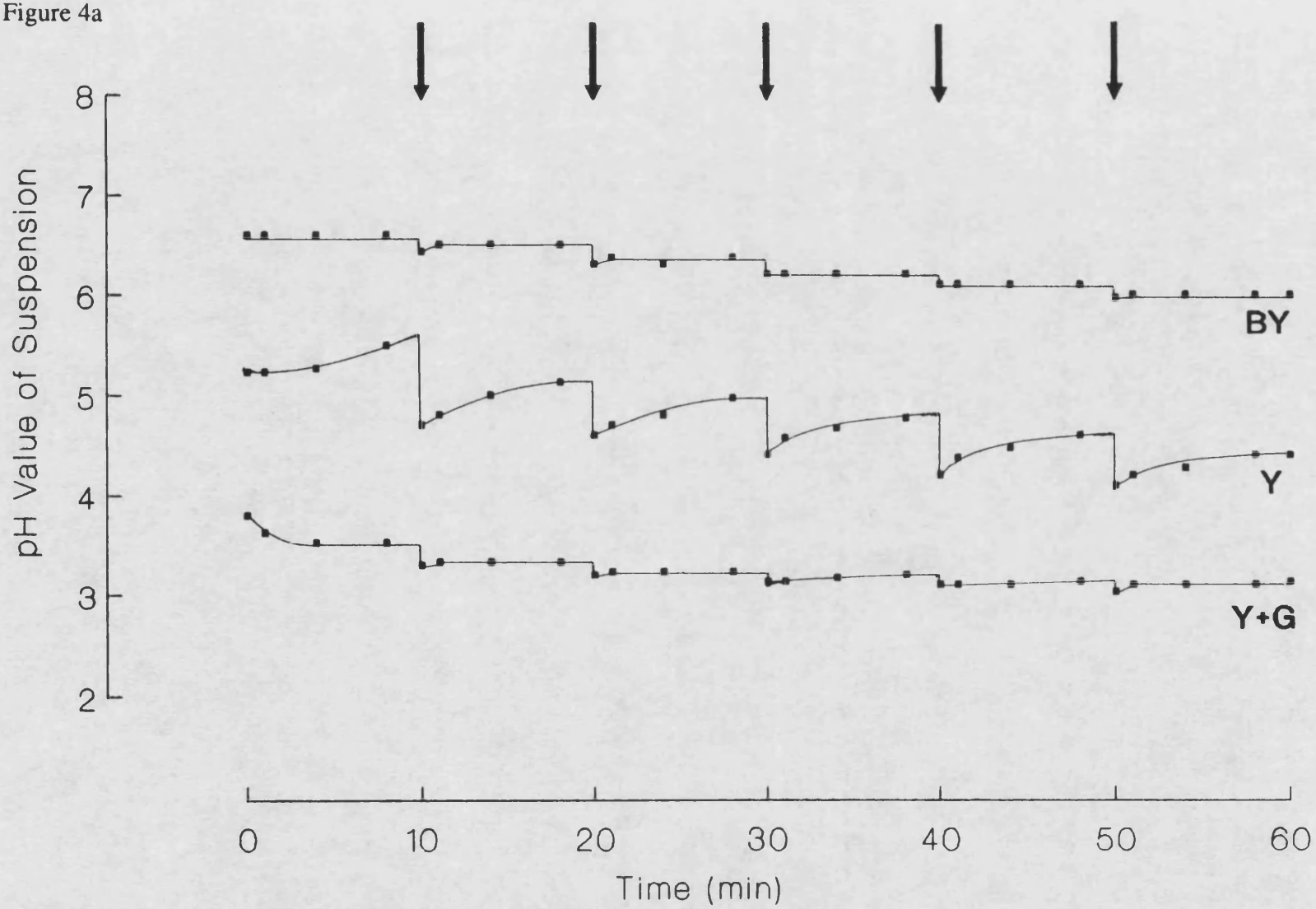

pH Value of Suspension
8
7
6
5
4
3
2
BY
Y
Y+G
0
10
20
30
40
50
60
Time (min)

Figure 4b

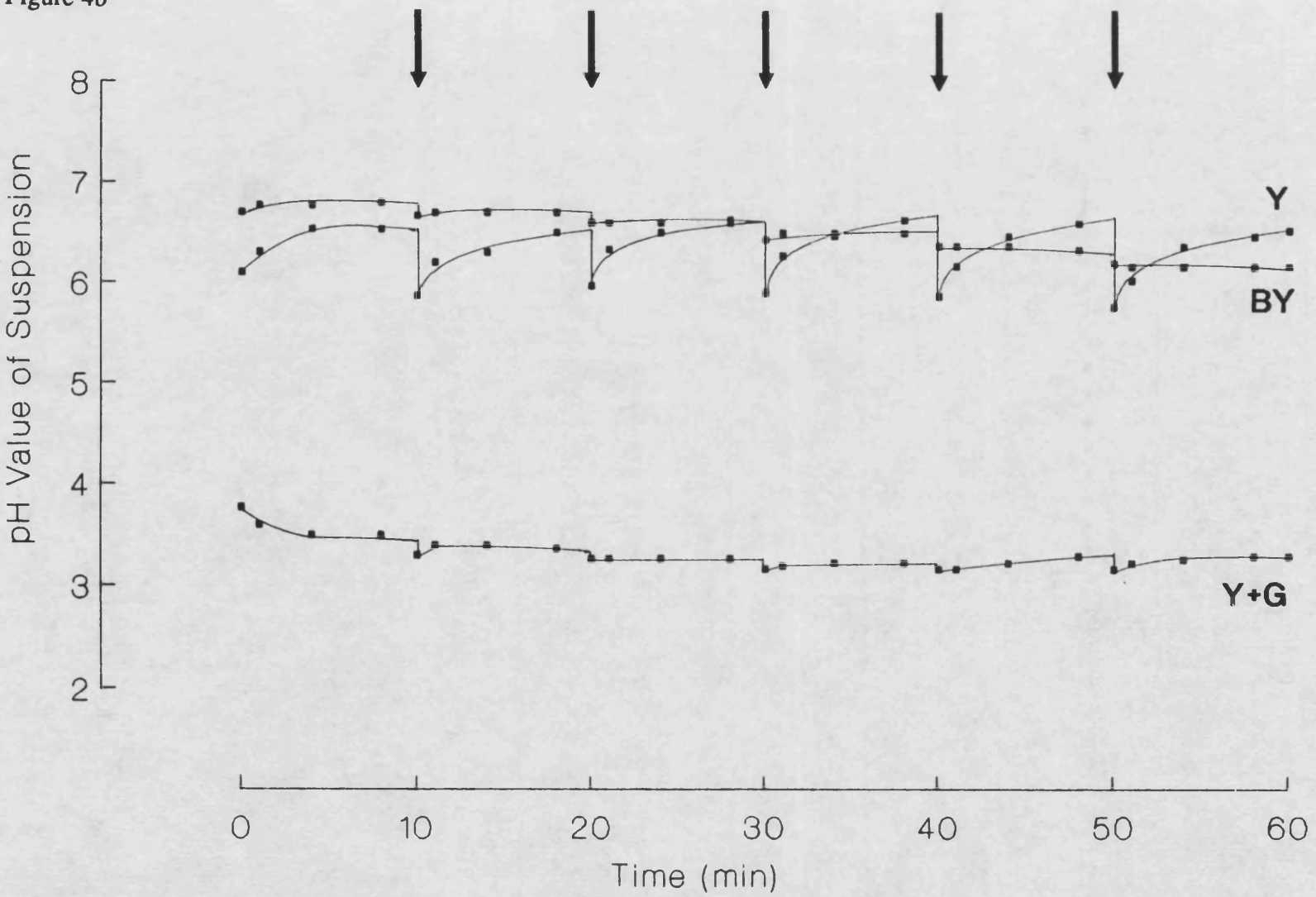

pH Value of Suspension
8
7
6
5
4
3
2
Y
BY
Y+G
0
10
20
30
40
50
60
Time (min)

Figure 4c

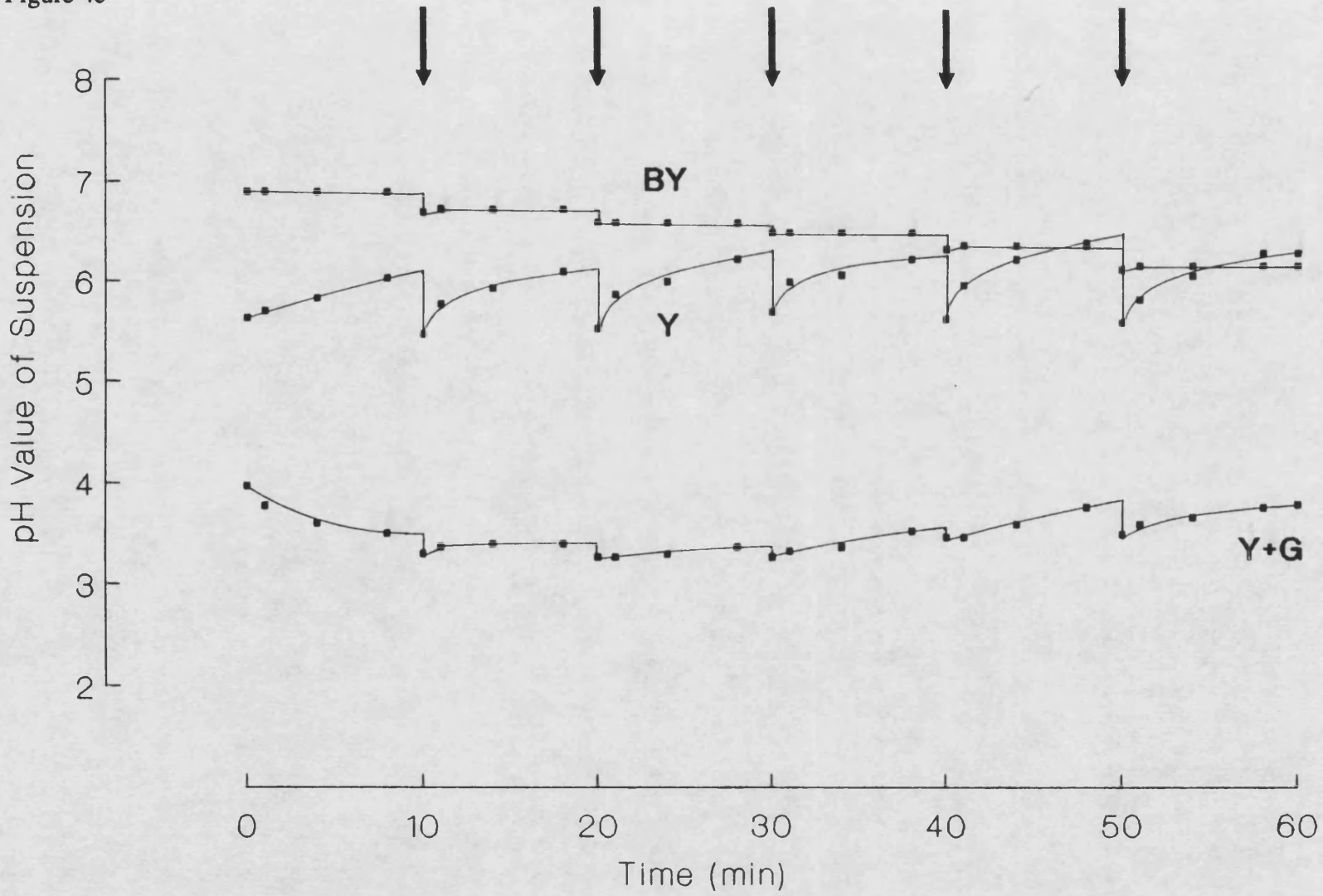
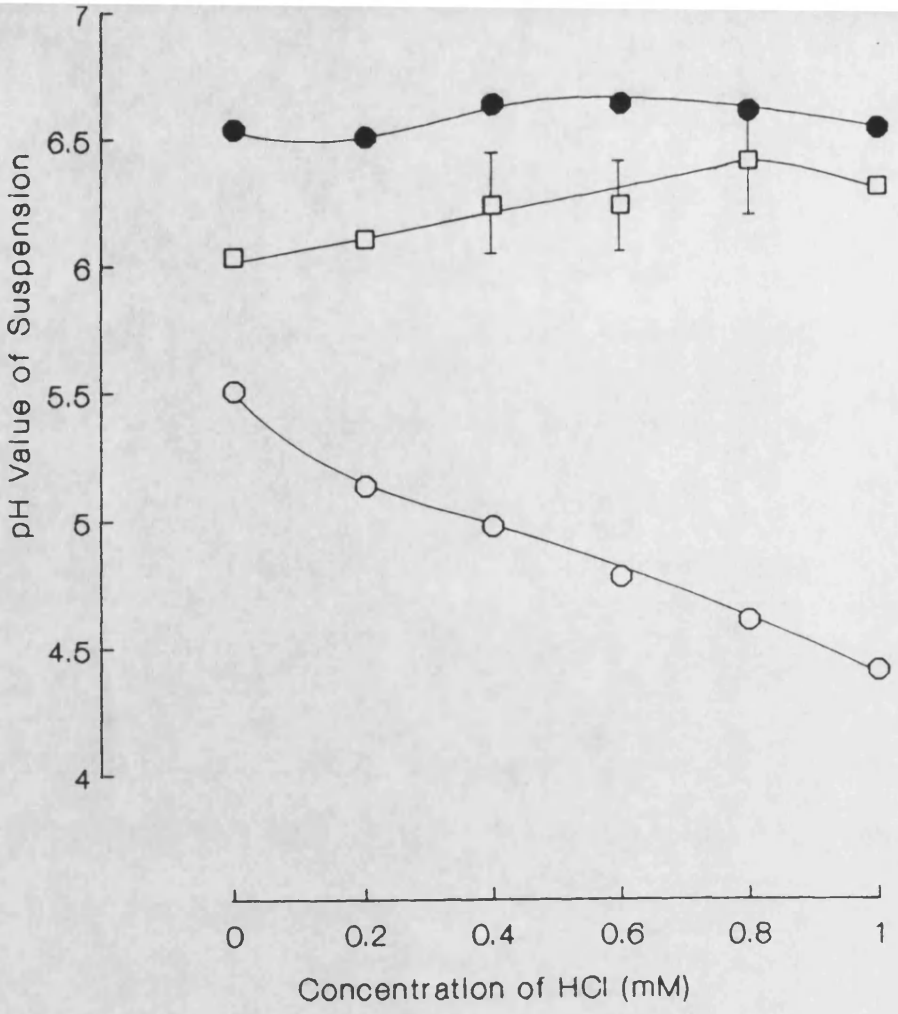
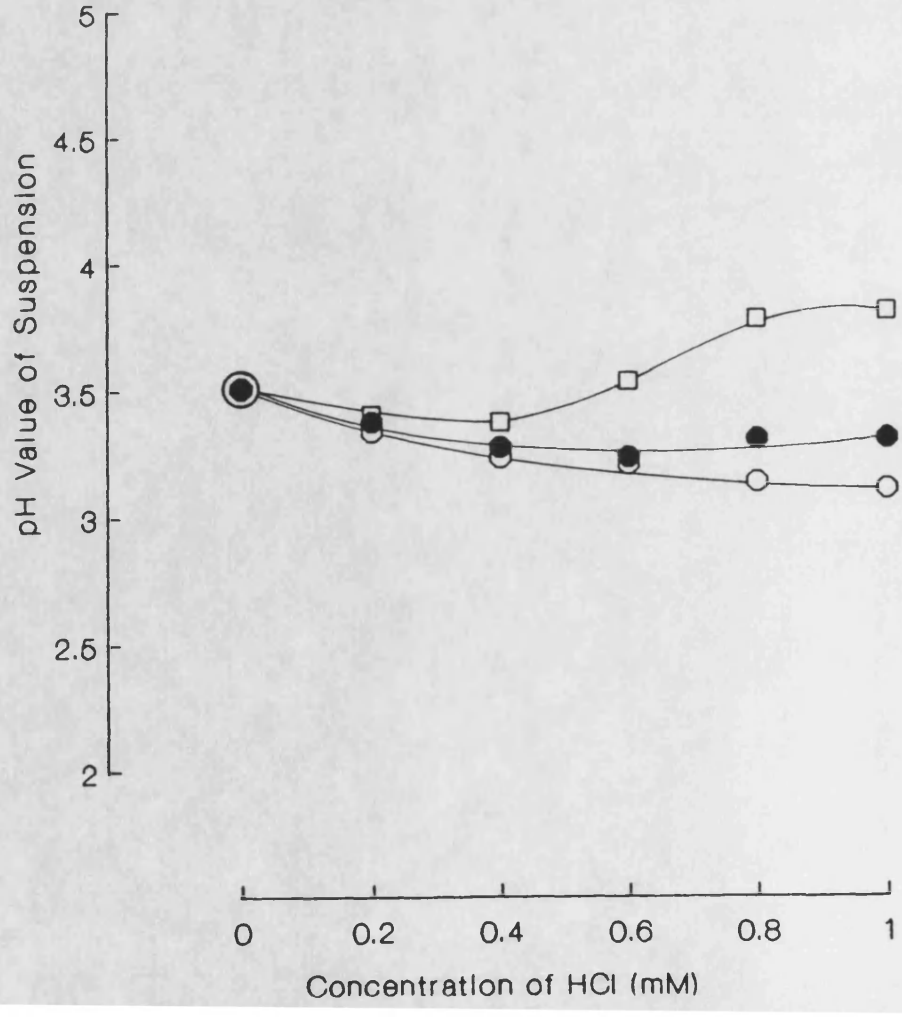

8
7
6
5
4
3
2
pH Value of Suspension
BY
Y
Y+G
0
10
20
30
40
50
60
Time (min)

Figure 5a


pH Value of Suspension
7
6.5
6
5.5
5
4.5
4
0
0.2
0.4
0.6
0.8
1
Concentration of HCl (mM)

5b


pH Value of Suspension
5
4.5
4
3.5
3
2.5
2
0
0.2
0.4
0.6
0.8
1
Concentration of HCl (mM)

5c

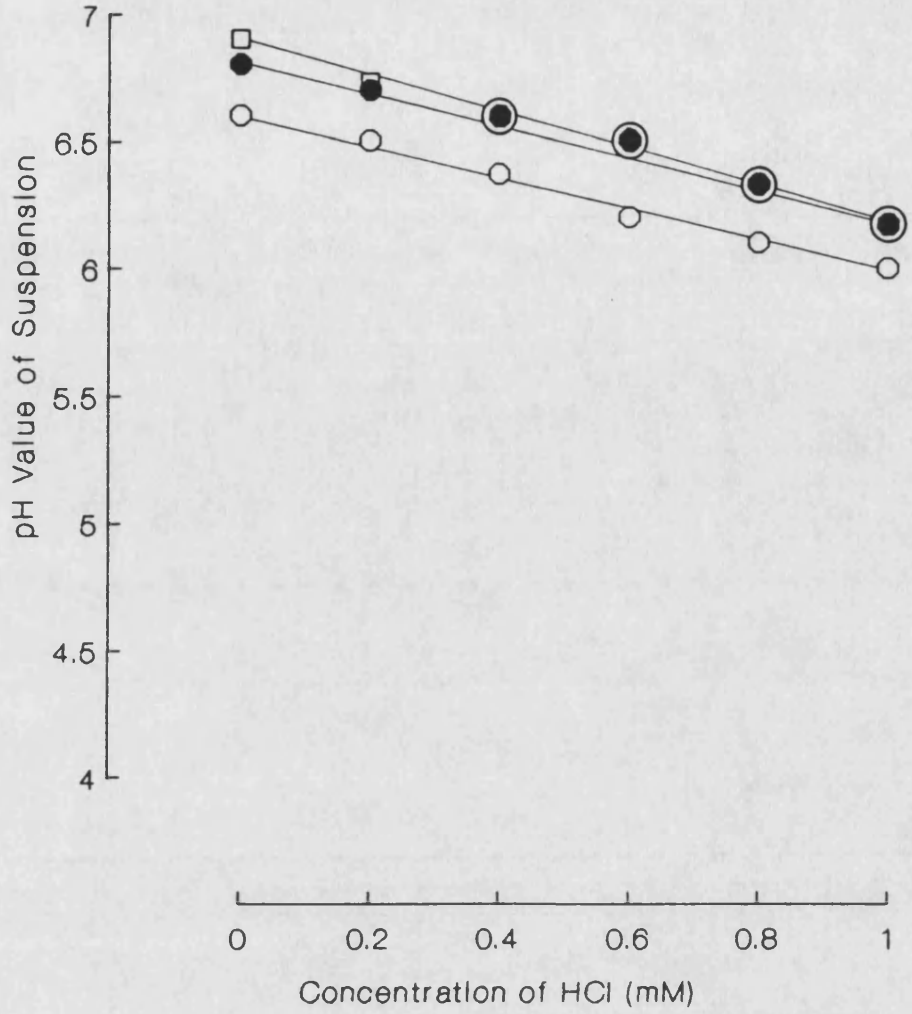


Figure 5. Final pH values following addition of hydrochloric acid (50 mM) to yeast suspension lacking glucose (Fig. 5a), suspension containing glucose (50 mM; Fig. 5b) or suspension of boiled cells lacking glucose (Fig. 5c) for *Saccharomyces cerevisiae* AWRI 1A65 (○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□). Suspensions were maintained at 30°C with stirring. Following each acid addition (approx. 0.2 mM), the pH value of the suspension was allowed to reach a constant level. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

time periods, whereas pH values of control suspensions (not shown), to which no acid was added, remained constant. On adding acid to suspensions of viable cells, pH values immediately decreased and then gradually increased to steady levels. This effect of pH overshoot was observed with all three yeasts, but both strains of *S'codes ludwigii* demonstrated a greater recovery of pH value than did *Sacch. cerevisiae* AWRI 1A65. *Saccharomycodes ludwigii* BC1 and *S'codes ludwigii* TC10 were able to resist pH decline, whereas *Sacch. cerevisiae* AWRI 1A65 was not (Fig. 5). Supplementation of viable yeast suspensions with glucose (50 mM) induced acidification, and an initial pH value of 3.5 was measured in suspensions of each of the three yeasts. On addition of acid to these suspensions, a steady decline of pH value was initially observed with all three yeasts. However, at acid concentrations of 0.4 mM and above, pH values of suspensions of *S'codes ludwigii* strains deviated from the steady decline which was observed with *Sacch. cerevisiae* AWRI 1A65. This effect was most evident with suspensions of *S'codes ludwigii* TC10, where an overall increase in pH value of 0.3 unit was observed. Supplementation of suspensions with 100 mM glucose did not alter the distinction between the species of *Sacch. cerevisiae* and *S'codes ludwigii* in terms of resistance to pH decline.

TITRATION OF YEAST CELL COMPONENTS AGAINST ACID.

Cell washings of *Sacch. cerevisiae* AWRI 1A65 and both *S'codes ludwigii* strains, when titrated against hydrochloric acid, produced identical titration curves (Fig. 6). Samples of each yeast were also examined for the presence of capsular material. India-ink stains of organisms, when viewed under the light microscope, did not reveal the presence of a capsule with any of the yeast species.

Isolated cell walls were prepared by first disrupting organisms by homogenization with glass beads, and recovered by centrifugation followed by

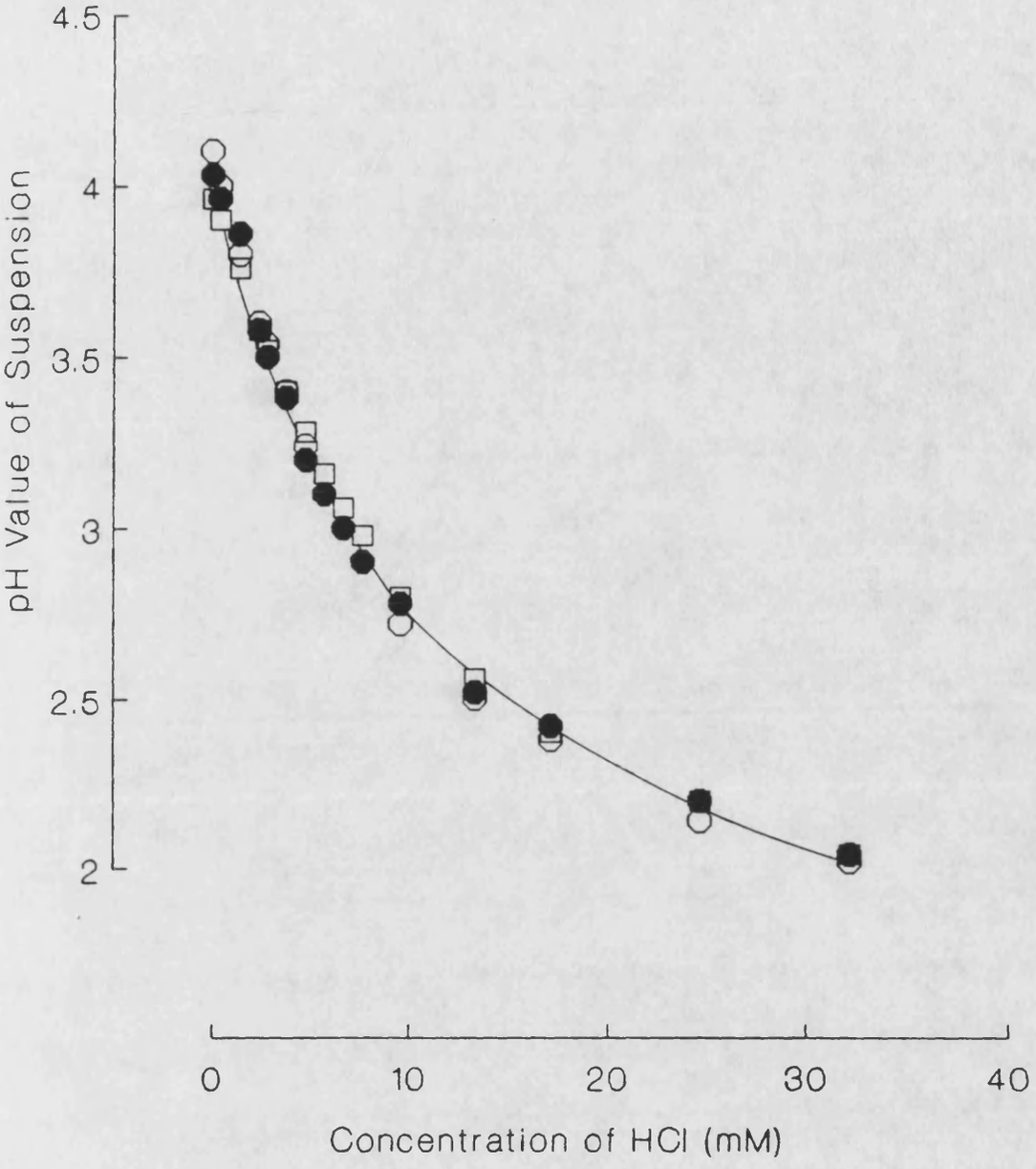


Figure 6. Final pH values following addition of hydrochloric acid (0.1 - 1.0 mM) to portions of cell washings from cultures of *Saccharomyces cerevisiae* AWRI 1A65 (○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□). Organisms (600 mg) were harvested in mid-exponential phase of growth, resuspended in 30 ml distilled water, and cell washings were recovered by centrifugation. Values given are the means of five independent determinations. Maximum S.D. for pH values was ± 0.25.

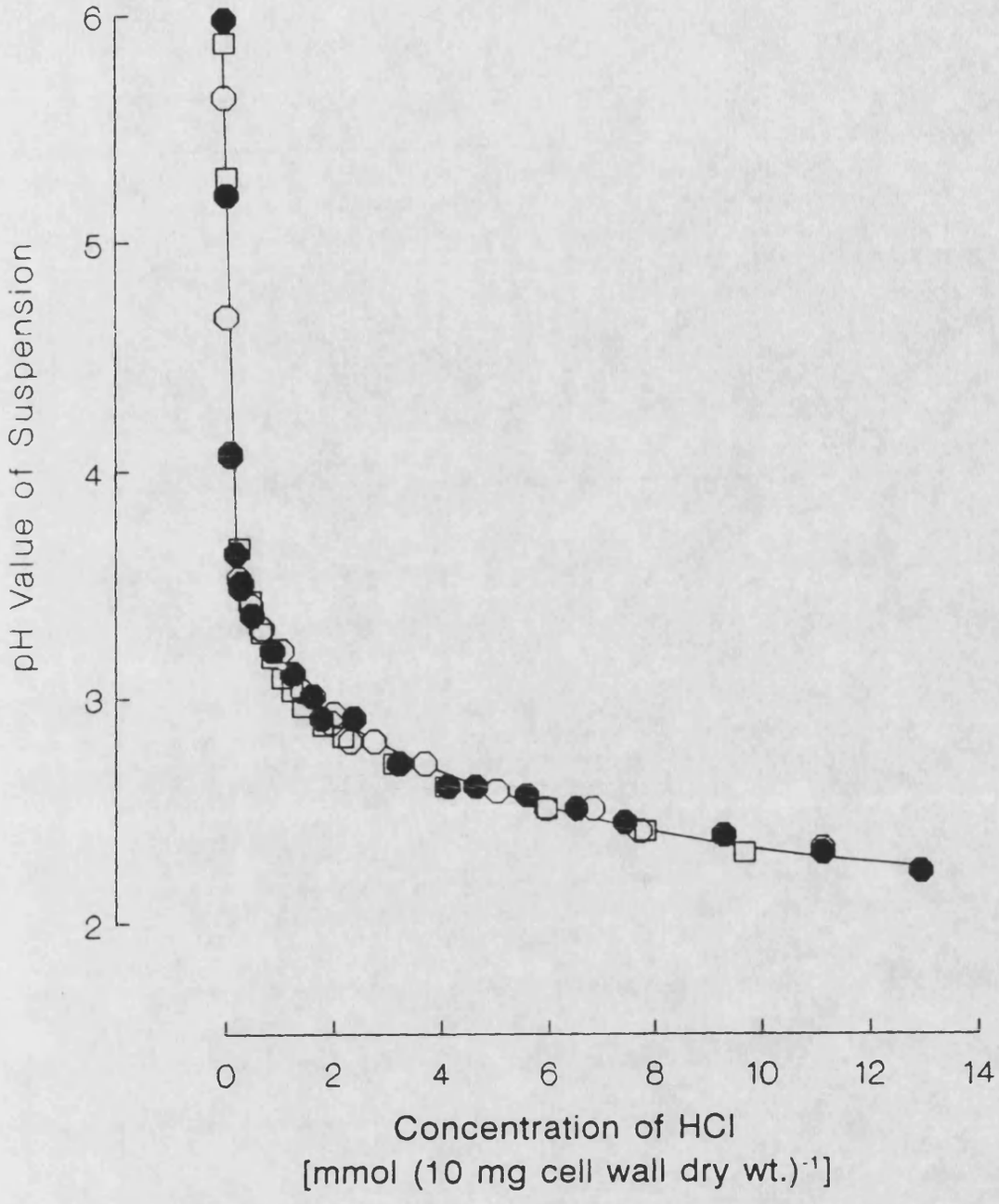


Figure 7. Final pH values following addition of portions of hydrochloric acid (5 - 250 mM) to suspensions of isolated cell walls from *Saccharomyces cerevisiae* AWRI 1A65 (○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□). Isolated cell-wall material had been freeze-dried and resuspended to 0.1% (w/v) in water. Values given are the means of six independent determinations. Maximum S.D. for pH values was ± 0.20.

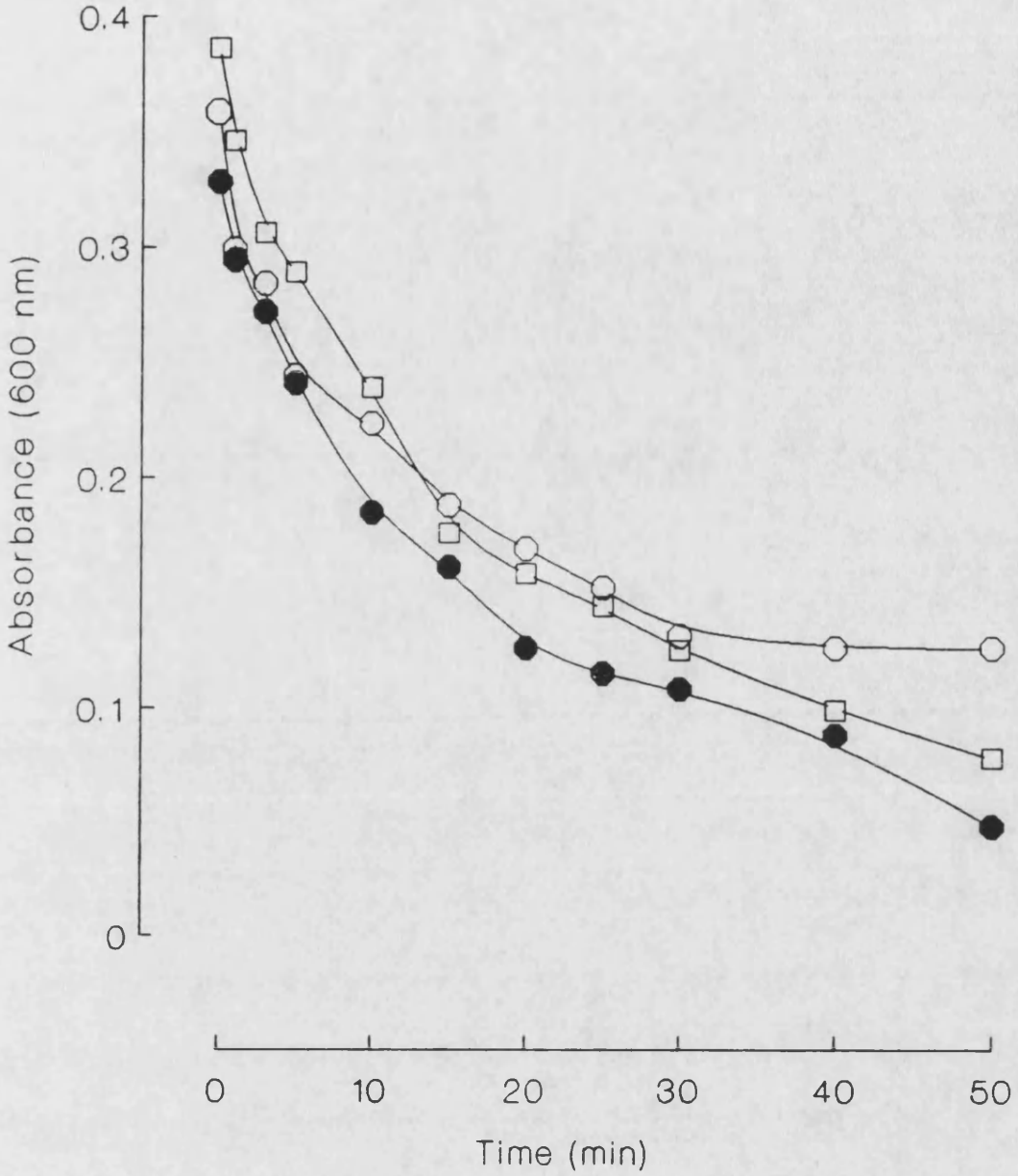


Figure 8. Time-course of absorbance (600 nm) decrease for samples, diluted 1 in 100 times in 2 M sorbitol, of unbuffered suspensions of *Saccharomyces cerevisiae* AWRI 1A65 (○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□), each at approx. 20 mg dry wt ml^{-1}, treated with ultrasound at 20 kHz. Values given are the means of at least two determinations. The maximum variation in values of absorbance was ± 0.02. In subsequent experiments, where organisms were sonicated in order to isolate cell cytosols, a treatment of duration 20 - 25 min was adopted.

	Cytosolic Buffering Capacity	
	μmol HCl (mg dry wt.)$^{-1}$	μmol HCl (μl cell cytosols)$^{-1}$
Saccharomyces cerevisiae AWRI 1A65	0.255 ± 0.041	0.216 ± 0.034
Saccharomycodes ludwigii BC1	0.268 ± 0.011	0.155 ± 0.006
Saccharomycodes ludwigii TC10	0.257 ± 0.018	0.112 ± 0.008

Table 2. Buffering capacities of cell cytosols (concentration of HCl required to lower the pH value by one unit) of *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii*. Cell contents were isolated following disruption of yeast cells by ultrasound, and were titrated against 20 mM hydrochloric acid. Values given are the means of at least five independent determinations ± S.D.

repeated washing. When suspended in water and titrated against hydrochloric acid, cell walls of each yeast strain gave identical titration curves (Fig. 7). Lipid-free cell-wall material produced, with all three yeasts, the same titration curve as that produced by lipid-containing cell-wall material.

Cytosolic contents were obtained by first disrupting yeast cells, using ultrasound, and contents were recovered by centrifugation. Absorbance (600 nm) values of suspensions of strains of *S'codes ludwigii* decreased to a greater extent than those of *Sacch. cerevisiae* AWRI 1A65 during treatment with ultrasound (Fig. 8). Isolated cell cytosols were titrated against hydrochloric acid to give values of cytosolic buffering capacity and, expressed on a mg dry wt. basis, these values did not differ significantly between yeast strains (Table 2). However, expressed in the physiologically more important terms of μmol HCl (μl cytosolic fluid)$^{-1}$ (pH unit decrease)$^{-1}$, *Sacch. cerevisiae* AWRI 1A65 was found to have the largest cytosolic buffering capacity, and *S'codes ludwigii* TC10 the smallest.

ACCUMULATION OF SULPHITE BY YEAST.

Unless otherwise stated, results of sulphite accumulation pertain to organisms which were harvested in the mid-exponential phase of growth.

Equilibrium levels for accumulation of sulphite were reached somewhat faster with strains of *S'codes ludwigii* than with *Sacch. cerevisiae* AWRI 1A65, although all three strains had reached equilibrium after about 6 min, irrespective of the concentration of sulphite (Fig. 9). At equilibrium, intracellular concentrations of sulphite were considerably higher in *Sacch. cerevisiae* AWRI 1A65 than in either strain of *S'codes ludwigii*; for the same extracellular concentration, *Sacch. cerevisiae* AWRI 1A65 accumulated ten times more sulphite than *S'codes ludwigii*.

Figure 9. Time-course of accumulation of sulphite by *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 9a), *Saccharomycodes ludwigii* BC1 (Fig. 9b) and *Saccharomycodes ludwigii* TC10 (Fig. 9c), each at 1.0 mg dry wt. ml^{-1}, from citric acid buffer (pH 4.0) containing glucose (100 mM), [^{35}S]sulphite (0.2 - 0.6 μCi ml^{-1}) and non-radioactive sulphite at 0.05 mM (○), 0.10 mM (●), 0.16 mM (□), 0.25 mM (■), 0.50 mM (△), 1.00 mM (▲) or 1.56 mM (◇). Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

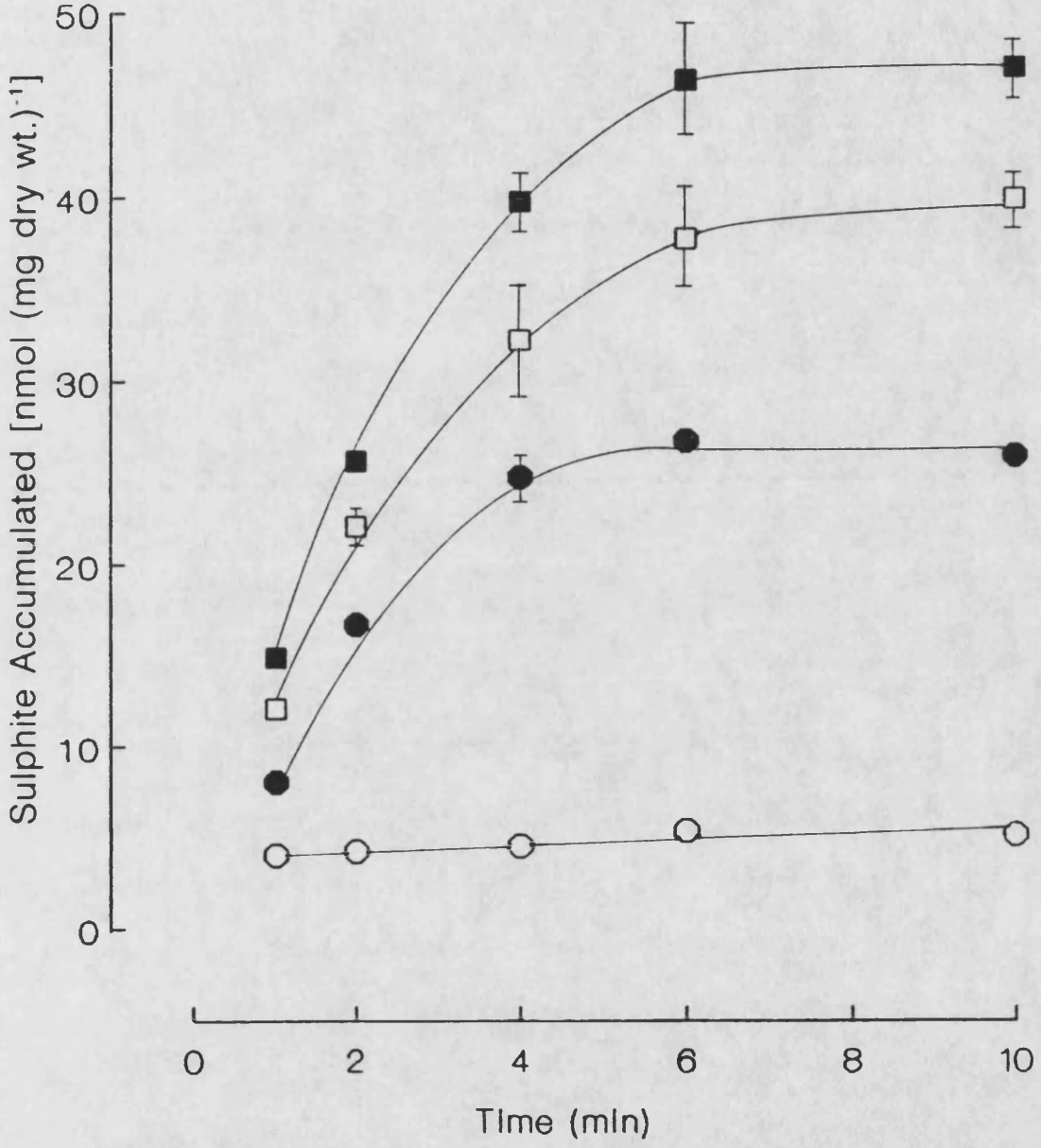

Sulphite Accumulated [nmol (mg dry wt.)-1]
50
40
30
20
10
0
0
2
4
6
8
10
Time (min)

Figure 9a

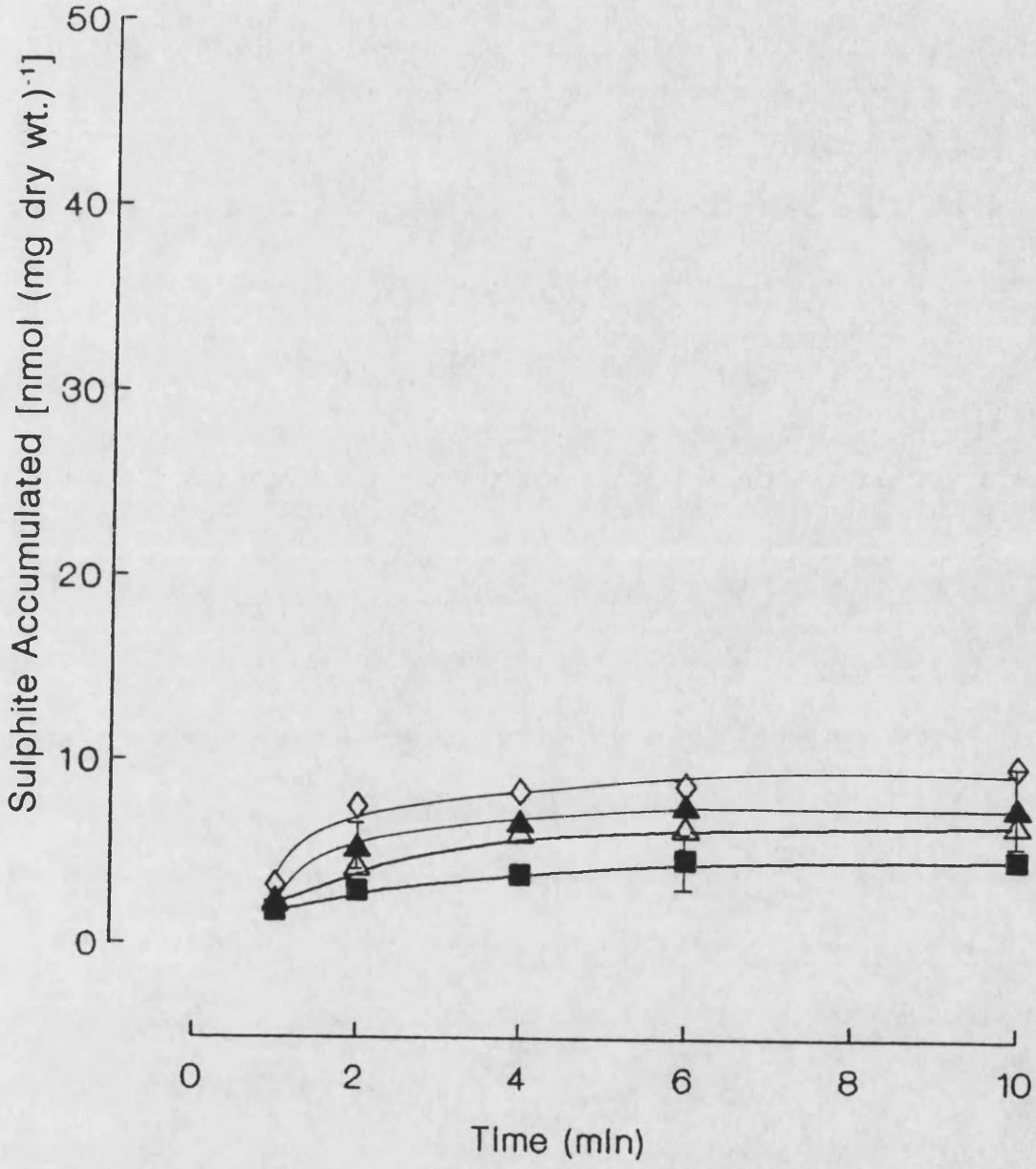

Sulphite Accumulated [nmol (mg dry wt.)-1]
50
40
30
20
10
0
0
2
4
6
8
10
Time (min)

Figure 9b

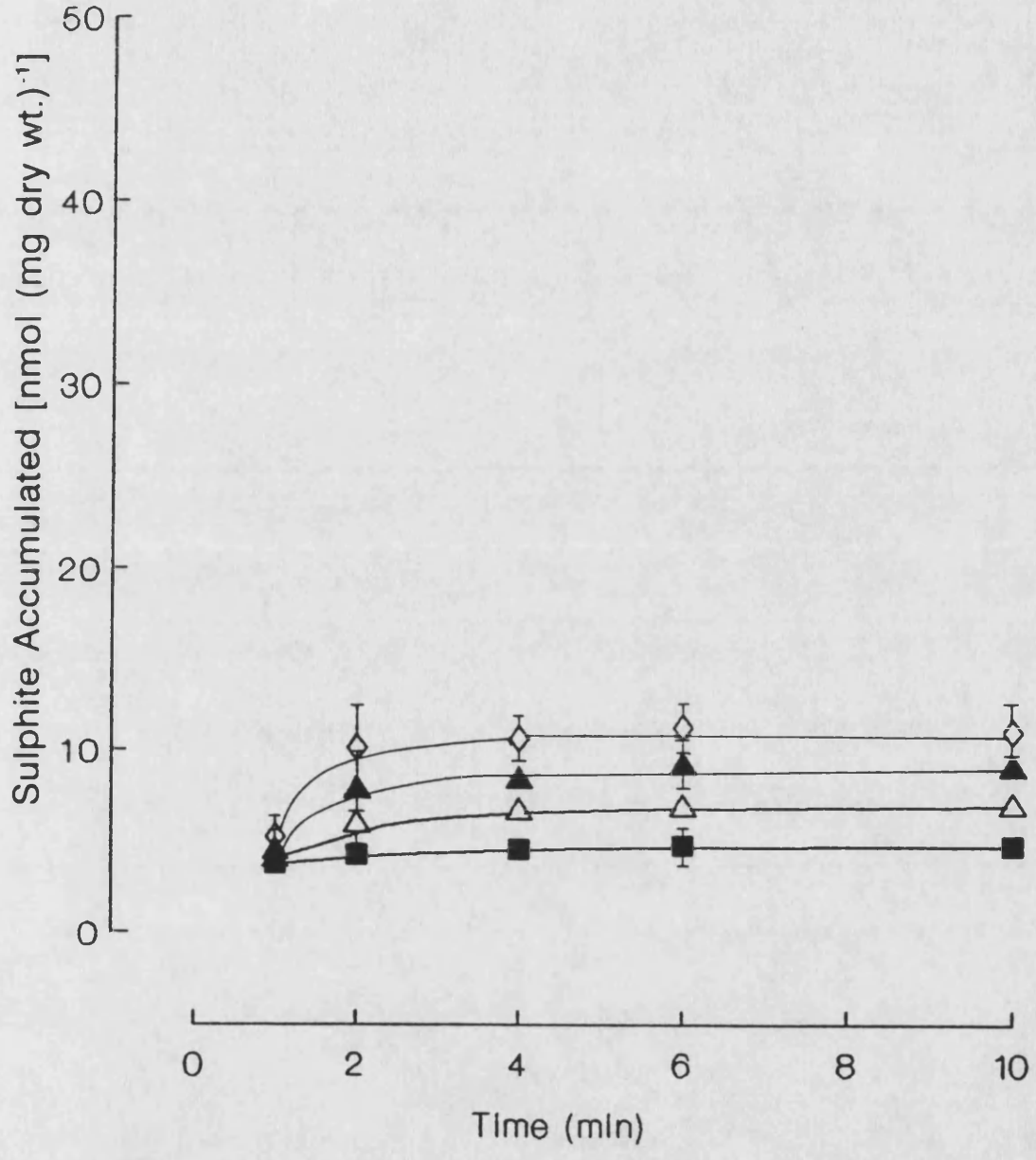

Sulphite Accumulated [nmol (mg dry wt.)-1]
50
40
30
20
10
0
0
2
4
6
8
10
Time (min)

Figure 9c

Maximum capacities for sulphite accumulation were established for all three yeast strains (Fig. 10). *Saccharomyces cerevisiae* AWRI 1A65, *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10 did not accumulate concentrations of sulphite greater than 97.6, 47.7 and 44.2 nmol (mg dry wt.)$^{-1}$, respectively. Taking into account intracellular volume, these maxima correspond to 82.8, 27.7 and 19.3 nmol (μl intracellular fluid)$^{-1}$, respectively. Accumulation of sulphite by *Sacch. cerevisiae* AWRI 1A65, up to saturation point, occurred linearly at a rate of 206.5 nmol (mg dry wt.)$^{-1}$ (mM total extracellular sulphite)$^{-1}$. Accumulation of sulphite by *S'codes ludwigii*, however, occurred in two phases. At relatively low extracellular concentrations of sulphite, intracellular sulphite concentrations increased linearly at rates of 5.6 and 6.1 nmol (mg dry wt.)$^{-1}$ (mM total extracellular sulphite)$^{-1}$, respectively, for *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10; at relatively high concentrations of sulphite, accumulation occurred at rates of 19.7 and 25.1 nmol (mg dry wt.)$^{-1}$ (mM total extracellular sulphite)$^{-1}$, respectively. Induction of the second phase of accumulation required a higher extracellular sulphite concentration with *S'codes ludwigii* BC1 than with *S'codes ludwigii* TC10.

Organisms, harvested in different growth phases, were found to accumulate sulphite to different extents (Fig. 11). As with mid-exponential phase organisms, those harvested in early-exponential and stationary phases reached equilibrium somewhat more rapidly with both strains of *S'codes ludwigii* than with *Sacch. cerevisiae* AWRI 1A65. At sub-lethal concentrations of sulphite, *Sacch. cerevisiae* AWRI 1A65 and *S'codes ludwigii* BC1 accumulated least sulphite when harvested in stationary phase, and most sulphite when harvested in mid-exponential phase. *Saccharomycodes ludwigii* TC10 also accumulated most sulphite when harvested at mid-exponential phase, but accumulated least during early-exponential phase. Only small differences in sulphite accumulation between early-exponential and stationary-phase of both *S'codes ludwigii* strains were measured. At higher sulphite

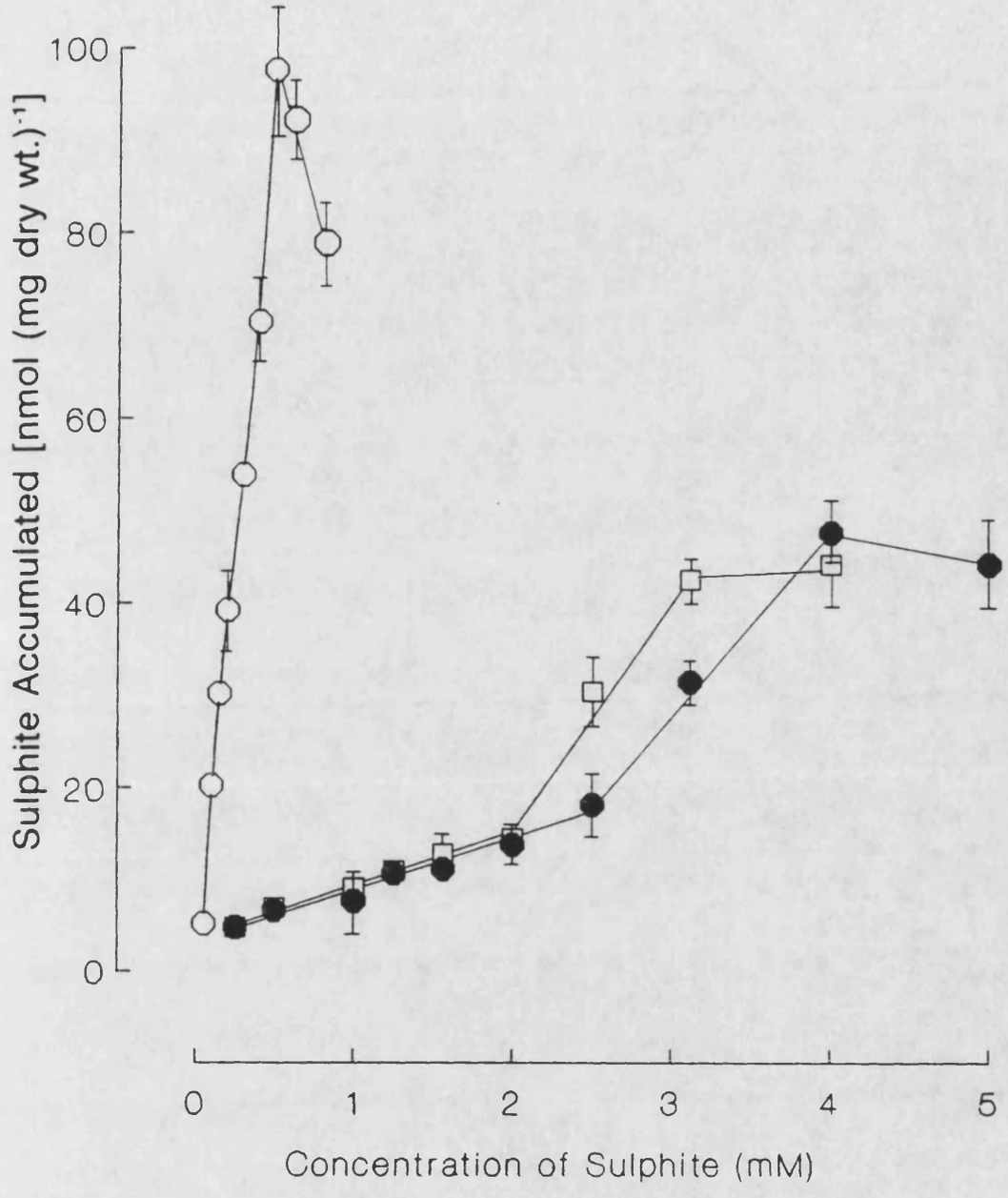
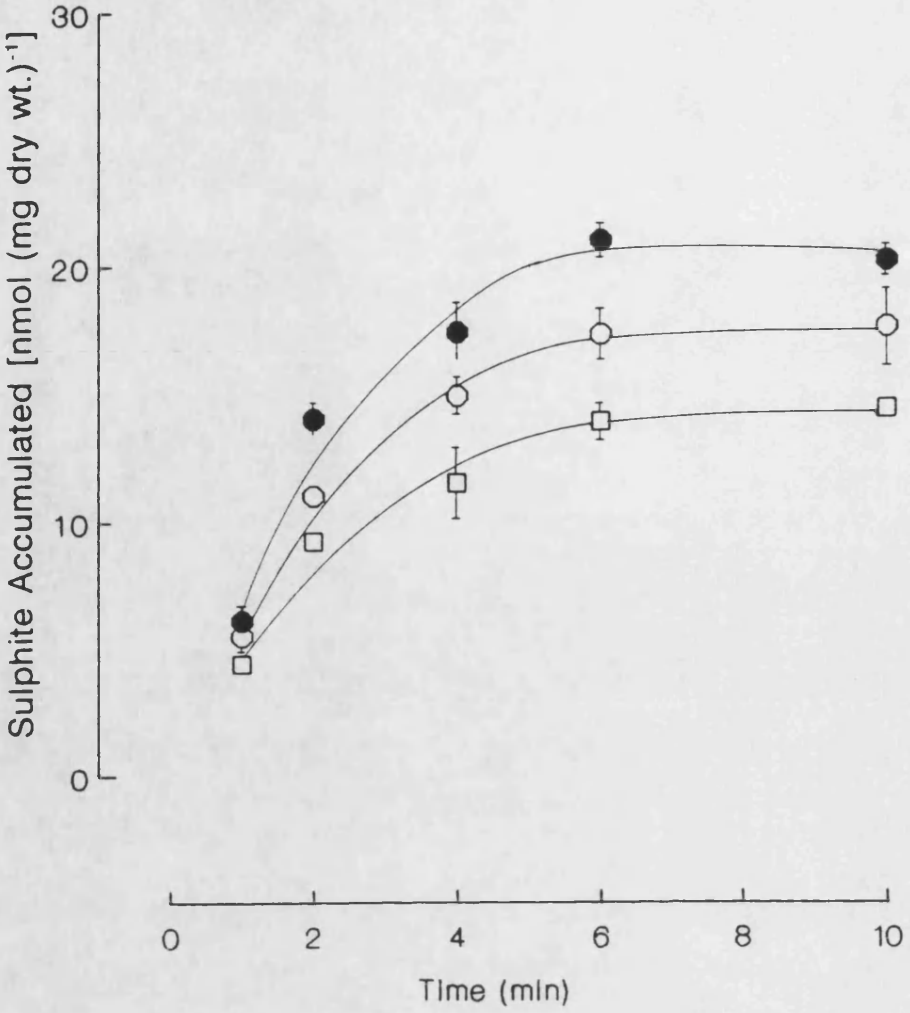
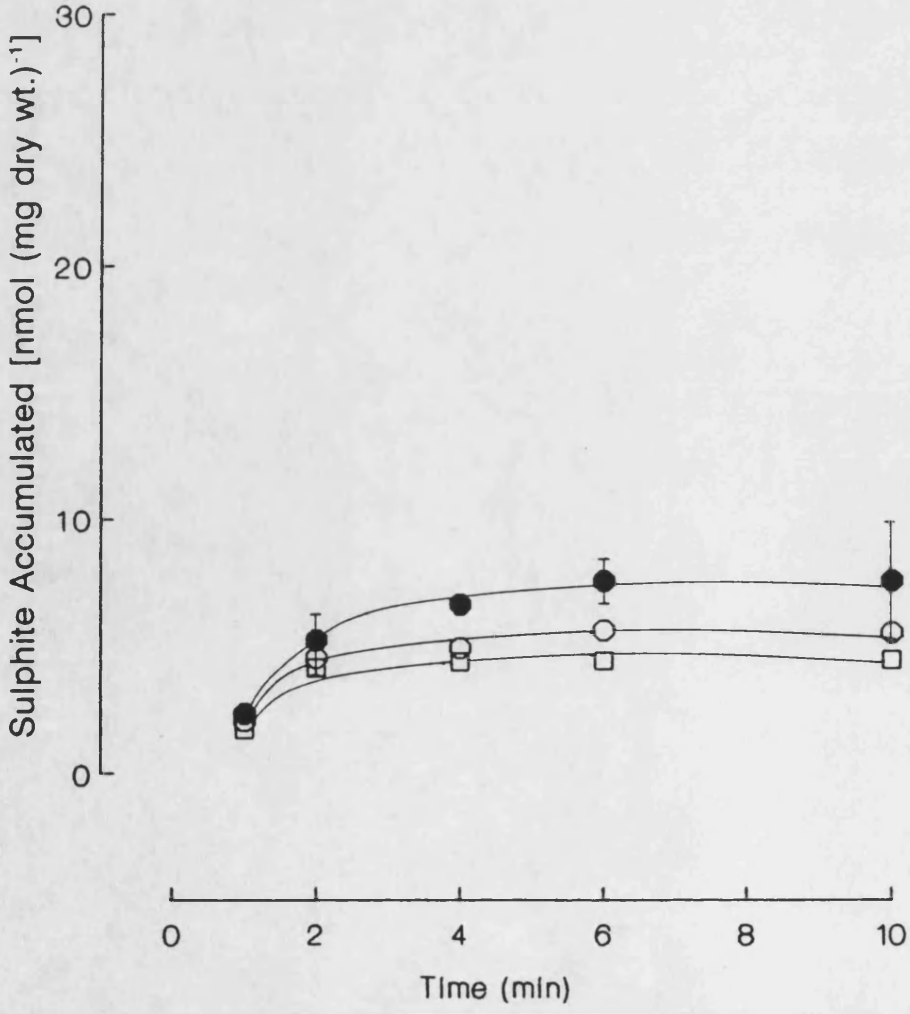


Figure 10. Accumulation of sulphite by *Saccharomyces cerevisiae* AWRI 1A65 (○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□). Measurements were made after organisms had been suspended to 1.0 mg dry wt. ml^{-1} in citric acid buffer (pH 4.0) containing glucose (100 mM), [^{35}S]sulphite (0.2 - 0.6 μCi ml^{-1}) and non-radioactive sulphite (0 - 5.0 mM) for 10 min. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

Figure 11a



11b



11c

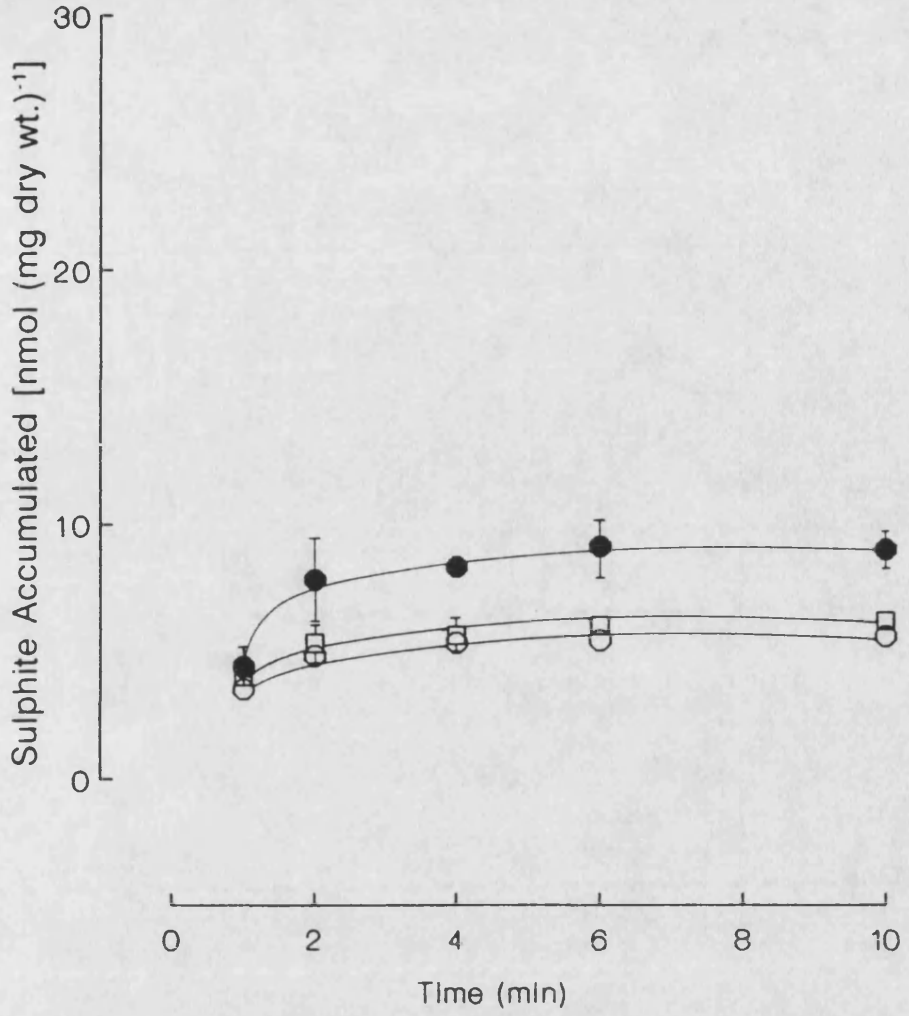
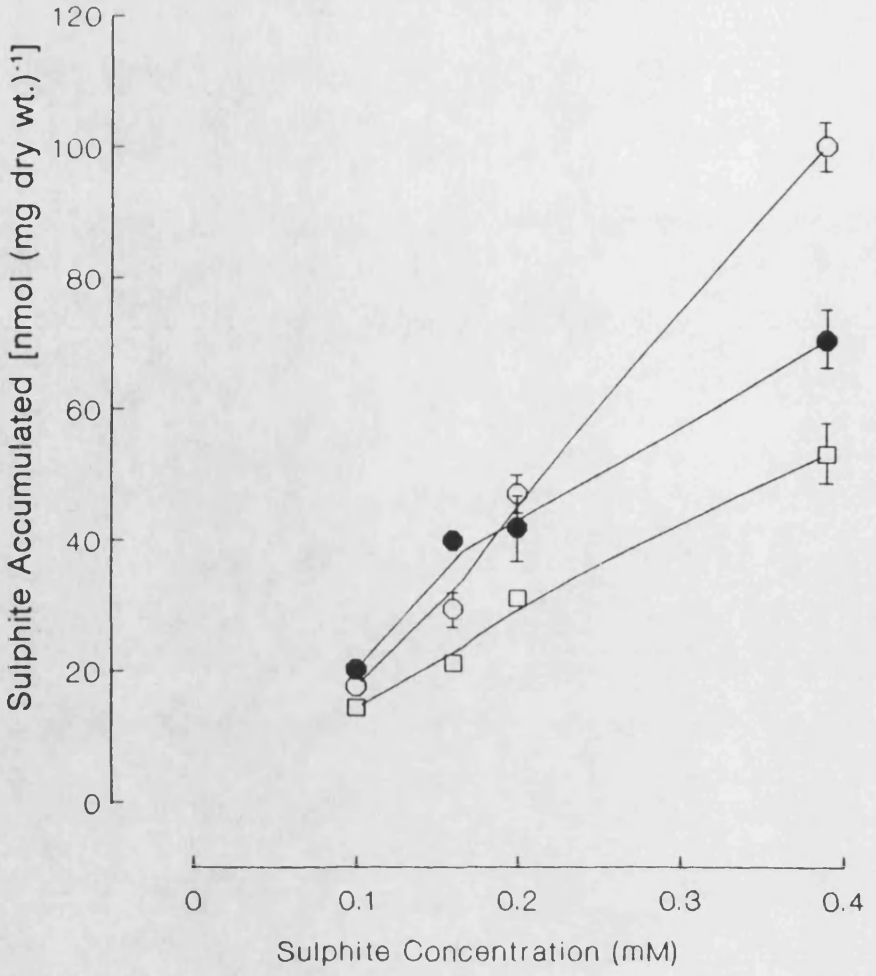
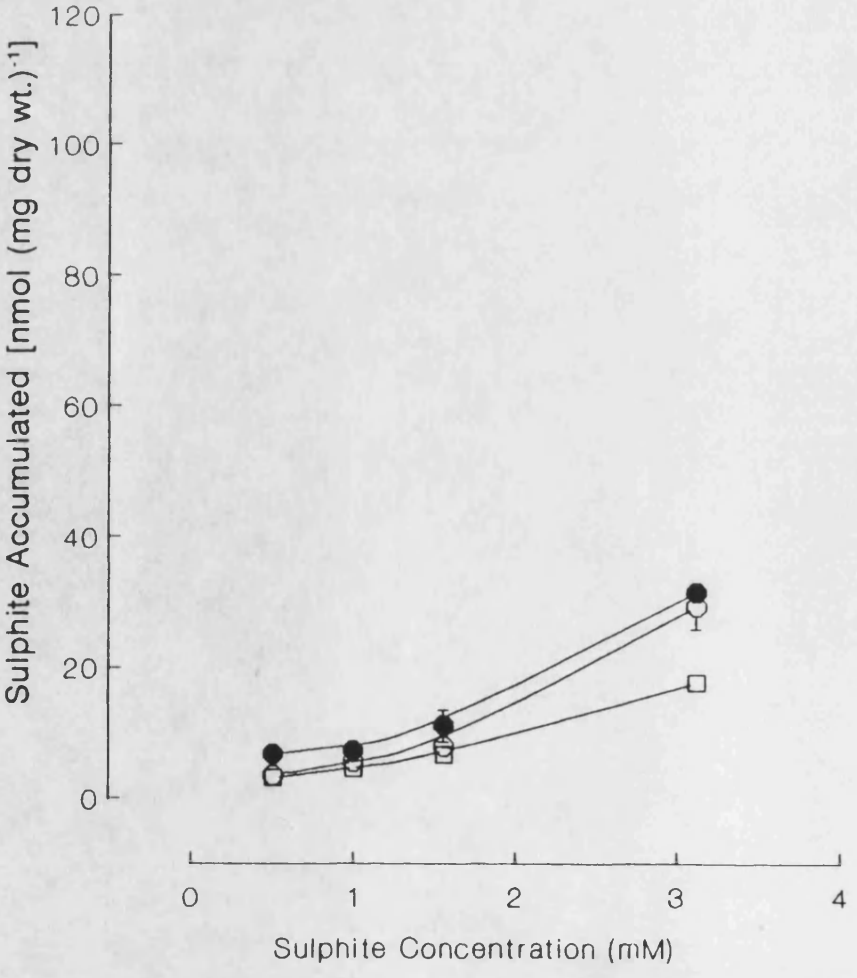


Figure 11. Time-course of accumulation of sulphite by *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 11a), *Saccharomycodes ludwigii* BC1 (Fig. 11b) and *Saccharomycodes ludwigii* TC10 (Fig. 11c) with respect to culture age. Organisms were harvested at early- (○) or mid- (●) exponential phases, or stationary (□) phase of growth. Measurements were made after organisms had been suspended to 1.0 mg dry wt. ml^{-1} in citric acid buffer (pH 4.0) containing glucose (100 mM), [^{35}S]sulphite (0.2 - 0.6 μCi ml^{-1}) and non-radioactive sulphite at 0.1 mM for *Saccharomyces cerevisiae* AWRI 1A65 and 1.0 mM for both *Saccharomycodes ludwigii* strains. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

Figure 12a



12b



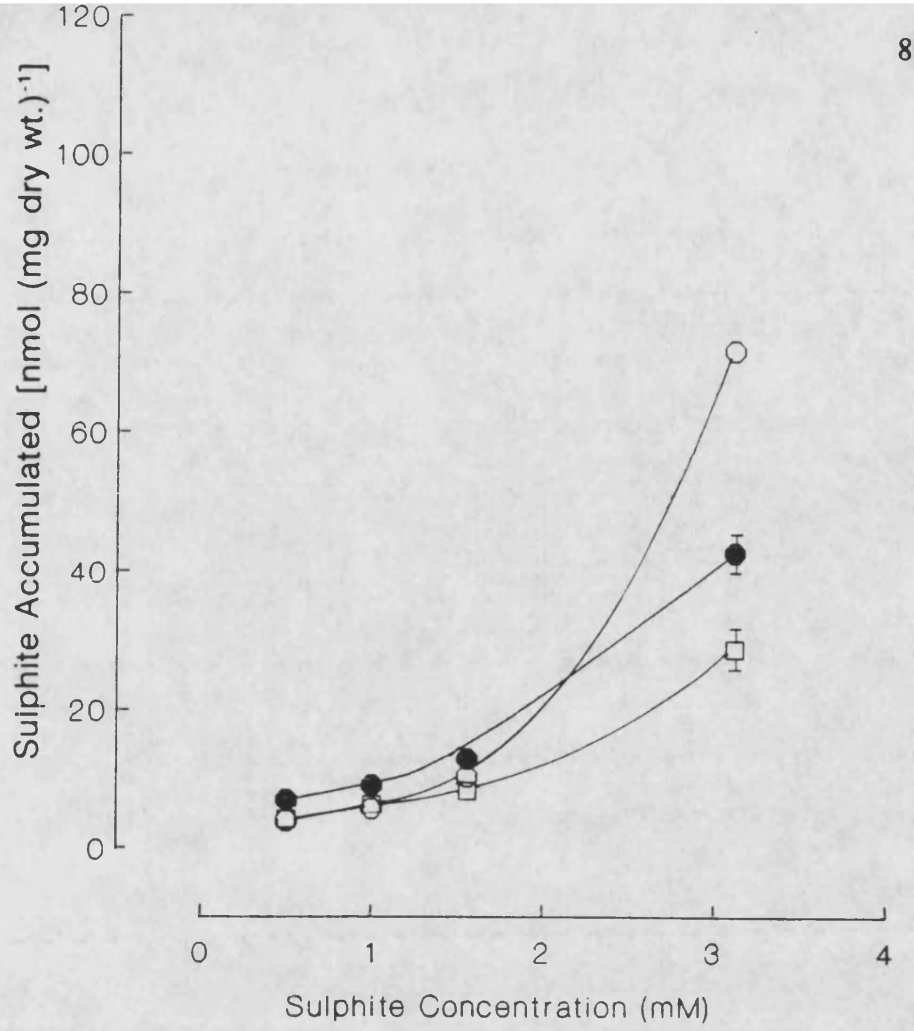


Figure 12. Accumulation of sulphite by *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 12a), *Saccharomycodes ludwigii* BC1 (Fig. 12b) and *Saccharomycodes ludwigii* TC10 (Fig. 12c) with respect to culture age. Organisms were harvested at early- (○) or mid- (●) exponential phases, or stationary (□) phase of growth. Measurements were made after organisms had been suspended to 1.0 mg dry wt. ml^{-1} in citric acid buffer (pH 4.0) containing glucose (100 mM), [^{35}S]sulphite (0.2 - 0.6 $\mu Ci\ ml^{-1}$) and non-radioactive sulphite (0 - 3.12 mM) for 10 min. Values given are the means of at least three independent determinations. Bars indicate SD., unless error lies within size of data point.

concentrations, *Sacch. cerevisiae* AWRI 1A65 and *S'codes ludwigii* TC10 accumulated greatest sulphite concentrations when harvested from the early-exponential phase of growth (Fig. 12).

INTRACELLULAR pH VALUES OF YEASTS.

Propionic acid was accumulated to different extents by each yeast strain, and at a range of concentrations depending upon the growth phase in which organisms were harvested (Fig. 13). Equilibrium levels of accumulation were reached after 6 - 8 min exposure to propionic acid. *Saccharomyces cerevisiae* AWRI 1A65 and *S'codes ludwigii* BC1 accumulated greatest concentrations of propionic acid when harvested in early-exponential phase, and least when harvested in the stationary phase of growth. In common with the other yeast strains, *S'codes ludwigii* TC10 accumulated least propionic acid when harvested in stationary phase, but accumulated equal concentrations when harvested in early- and mid-exponential phases of growth.

The mean intracellular pH values of *Sacch. cerevisiae* AWRI 1A65, harvested in any phase of growth, were found to be higher than those of both *S'codes ludwigii* strains (Table 3). Harvested in the mid-exponential phase of growth, *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10 were found to have identical intracellular pH values; harvested in early-exponential and stationary phases, the latter yeast was found to have lower intracellular pH values than the former. The growth phase in which intracellular pH values were lowest, with all three strains, was stationary phase.

EFFECTS OF SULPHITE ON INTRACELLULAR pH VALUES.

With all three strains, as sulphite was accumulated, intracellular pH values were found to decline (Fig. 14). *Saccharomyces cerevisiae* AWRI 1A65

Figure 13. Time-course of accumulation of propionic acid by *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 13a), *Saccharomycodes ludwigii* BC1 (Fig. 13b) and *Saccharomycodes ludwigii* TC10 (Fig. 13c) with respect to culture age. Organisms were harvested at early- (○) or mid- (●) exponential phases, or stationary (□) phase of growth. Measurements were made after organisms had been suspended to 1.0 mg dry wt. ml^{-1} in citric acid buffer (pH 4.0) containing glucose (100 mM), [2-^{14}C]propionic acid (0.5 μCi ml^{-1}) and non-radioactive propionic acid (2 μM). Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

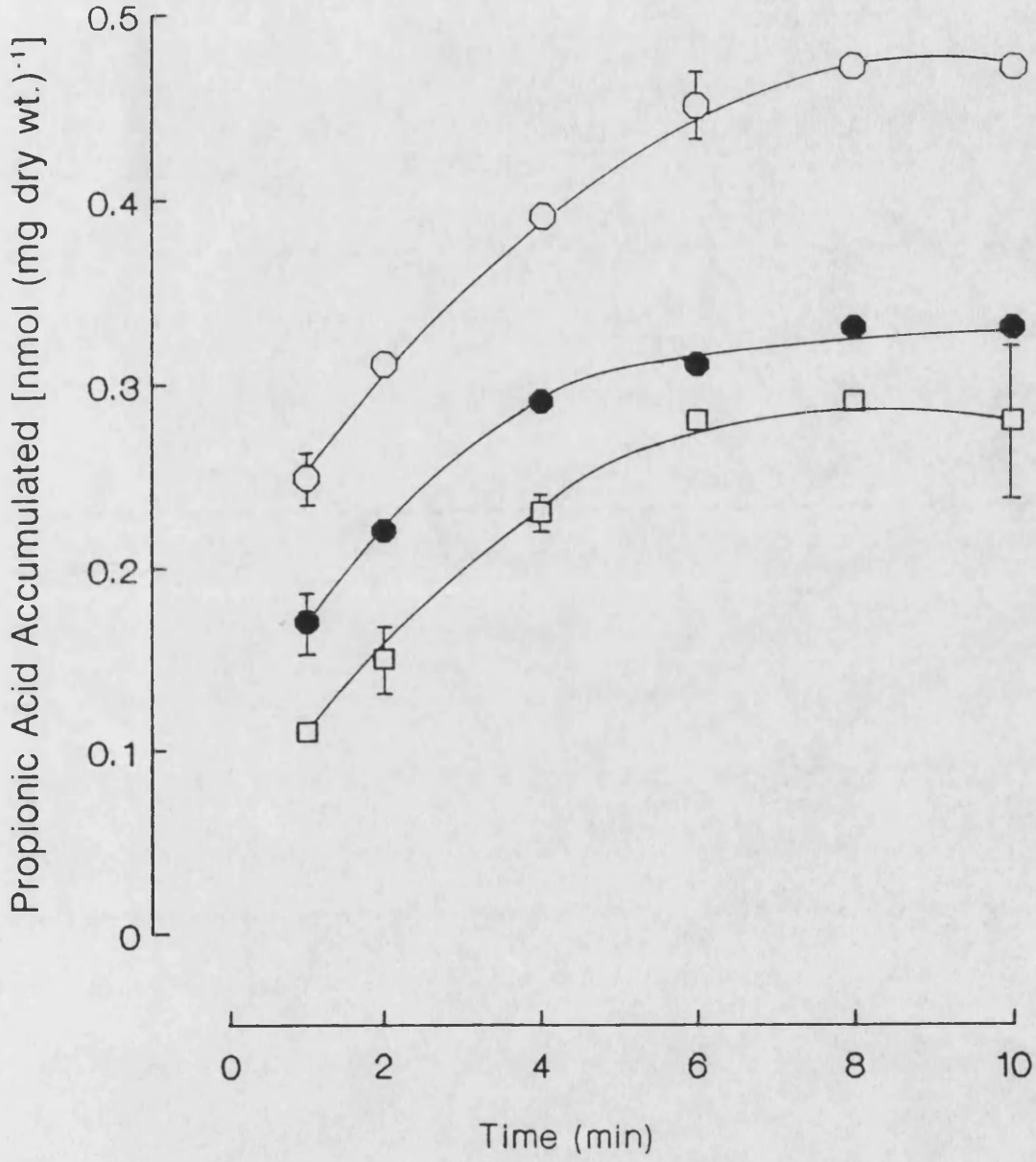

Propionic Acid Accumulated [nmol (mg dry wt.)⁻¹]
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
Time (min)

Figure 13a

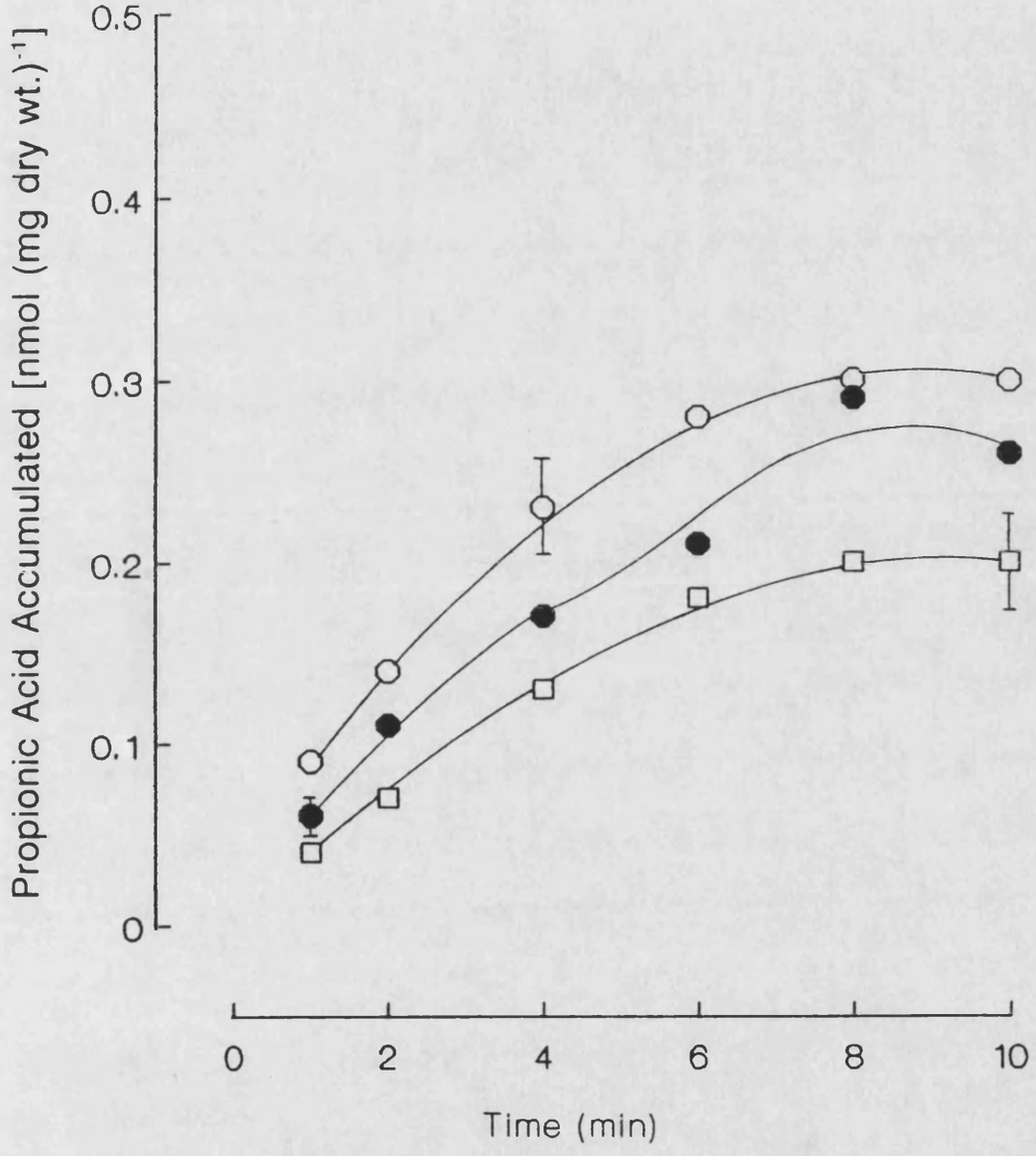

Propionic Acid Accumulated [nmol (mg dry wt.)$^{-1}$]
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
Time (min)

Figure 13b

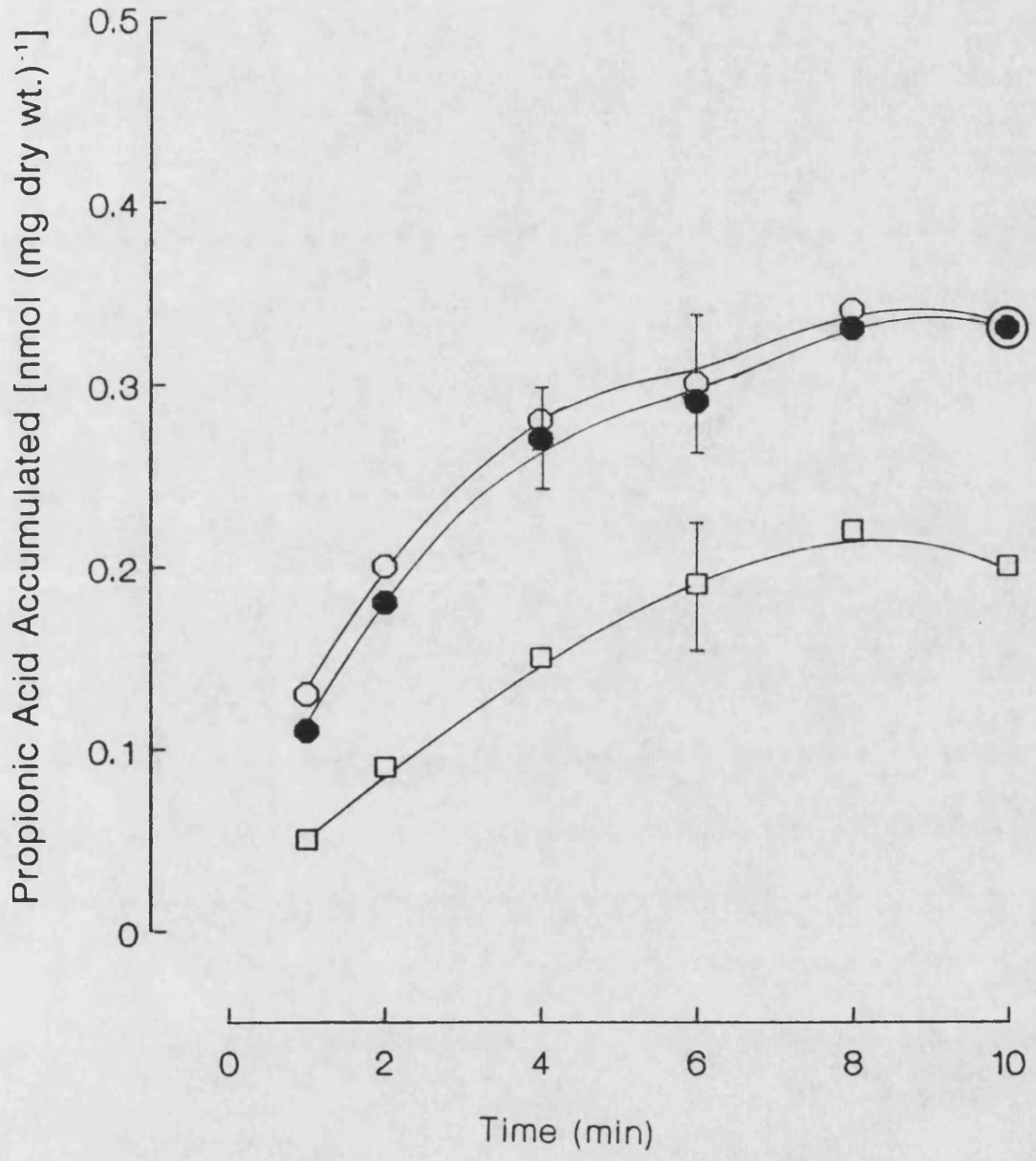

Propionic Acid Accumulated [nmol (mg dry wt.)$^{-1}$]
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
Time (min)

Figure 13c

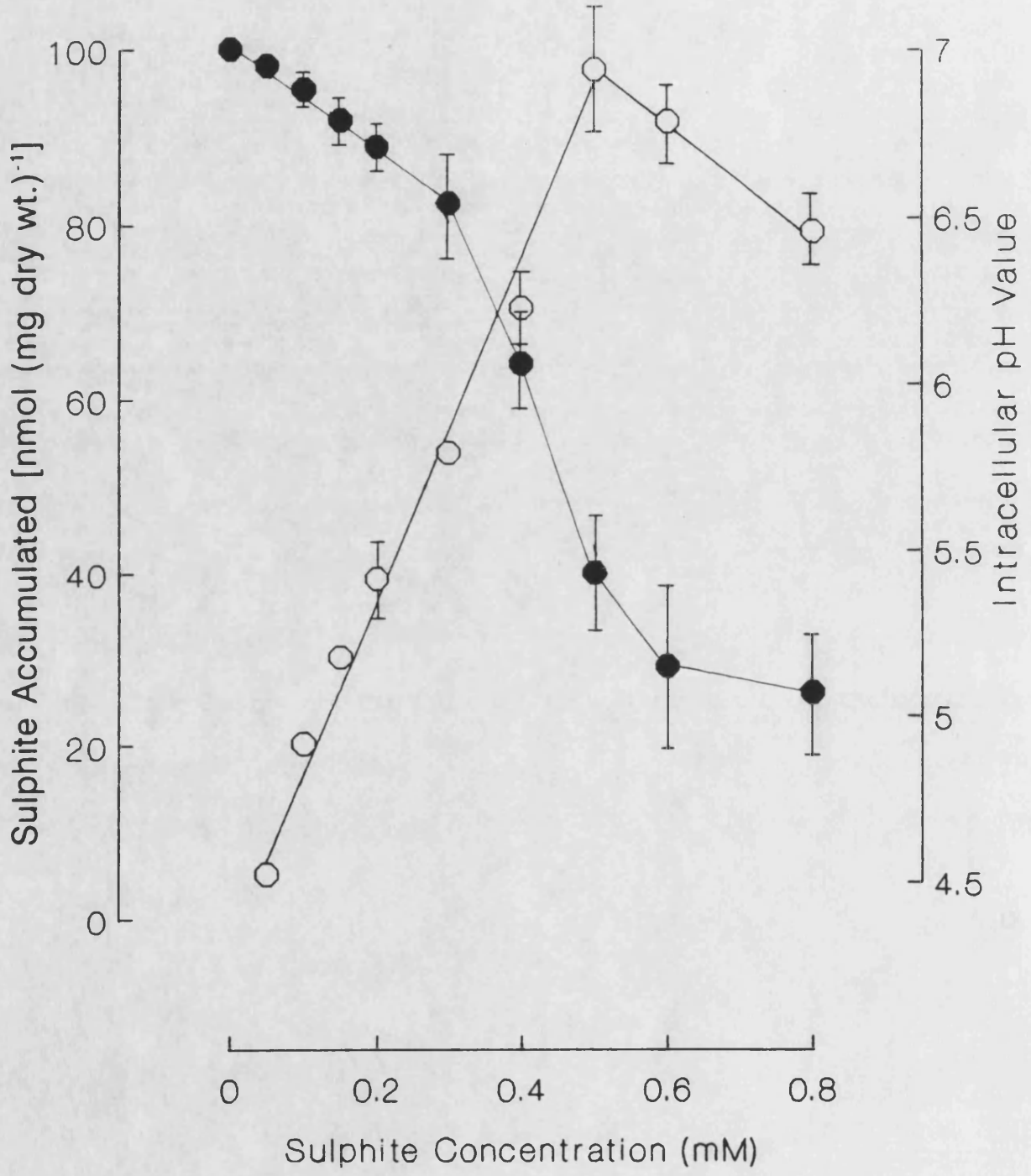
	Intracellular pH Value		
	Growth Phase From Which Organisms Were Harvested		
	Early-Exponential	Mid-Exponential	Stationary
Saccharomyces cerevisiae AWRI 1A65	7.22 ± 0.03	7.01 ± 0.03	6.93 ± 0.09
Saccharomycodes ludwigii BC1	6.81 ± 0.01	6.74 ± 0.02	6.61 ± 0.06
Saccharomycodes ludwigii TC10	6.73 ± 0.06	6.74 ± 0.03	6.49 ± 0.07

Table 3. Intracellular pH values of *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii* harvested in early- or mid-exponential phases, or stationary phase of growth. Values quoted were calculated from propionic acid-uptake data, and are the means of at least three independent determinations ± S.D.

Figure 14. (i) Relationship between extent of accumulation of sulphite (○) and change in intracellular pH values (●) of *Saccharomyces cerevisiae* AWRI 1A65 [Fig. 14a (i)], *Saccharomycodes ludwigii* BC1 [Fig. 14b (i)] and *Saccharomycodes ludwigii* TC10 [Fig. 14c (i)]. Measurements were made after organisms (1 mg dry wt. ml^{-1}) had been suspended for 10 min in citric acid buffer (pH 4.0) containing glucose (100 mM) and either radioactively-labelled sulphite (0 - 5 mM; 0.2 - 0.6 μCi ml^{-1}) or non-radioactive sulphite (0 - 5 mM) supplemented with radioactively labelled propionic acid (2 μM; 0.5 μCi ml^{-1}). Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

(ii) Effect of sulphite concentration on viability of *Saccharomyces cerevisiae* AWRI 1A65 [Fig. 14a (ii)], *Saccharomycodes ludwigii* BC1 [Fig. 14b (ii)] and *Saccharomycodes ludwigii* TC10 [Fig. 14c (ii)] suspended in citric acid buffer containing non-radioactive sulphite (0 - 5 mM) for 50 min. Values given are the means of at least three independent determinations. Error bars indicate S.D.

Figure 14a(i)


Sulphite Accumulated [nmol (mg dry wt.)$^{-1}$]
100
80
60
40
20
0
7
6.5
6
5.5
5
4.5
Intracellular pH Value
0
0.2
0.4
0.6
0.8
Sulphite Concentration (mM)

14a(ii)

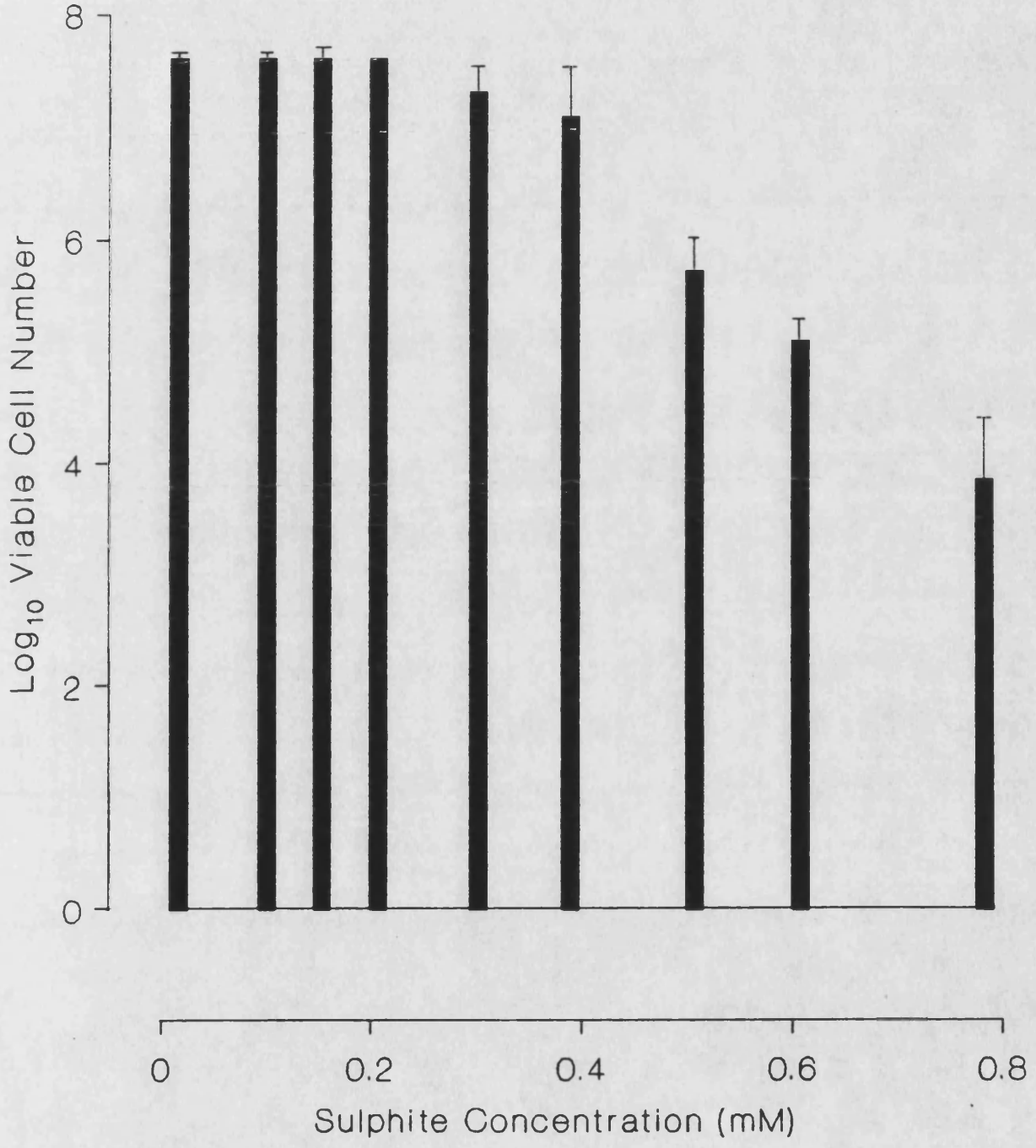
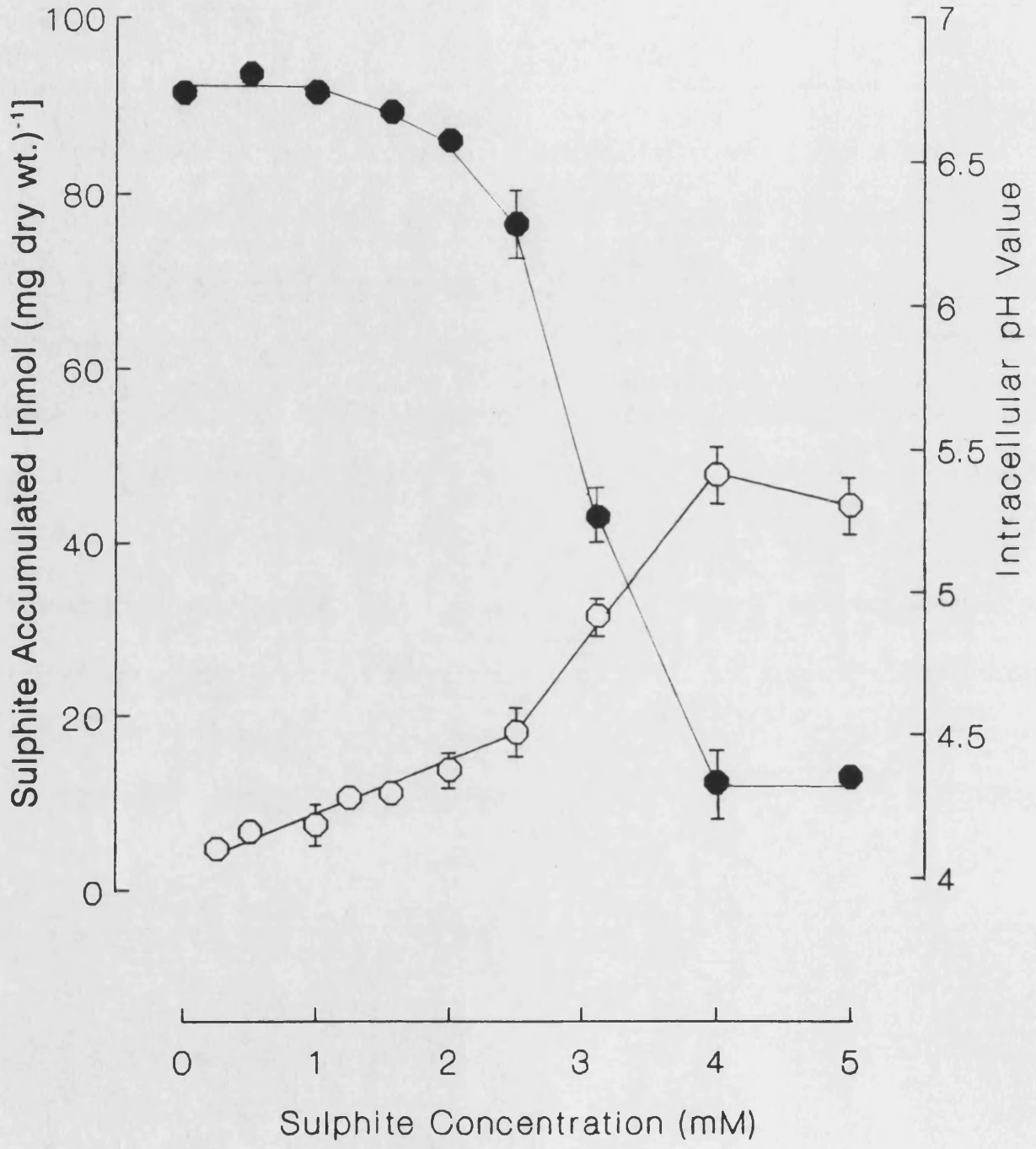

8
6
4
2
0
Log10 Viable Cell Number
0
0.2
0.4
0.6
0.8
Sulphite Concentration (mM)

Figure 14b(i)


Sulphite Accumulated [nmol (mg dry wt.)-1]
100
80
60
40
20
0
7
6.5
6
5.5
5
4.5
4
Intracellular pH Value
0
1
2
3
4
5
Sulphite Concentration (mM)

14b(ii)

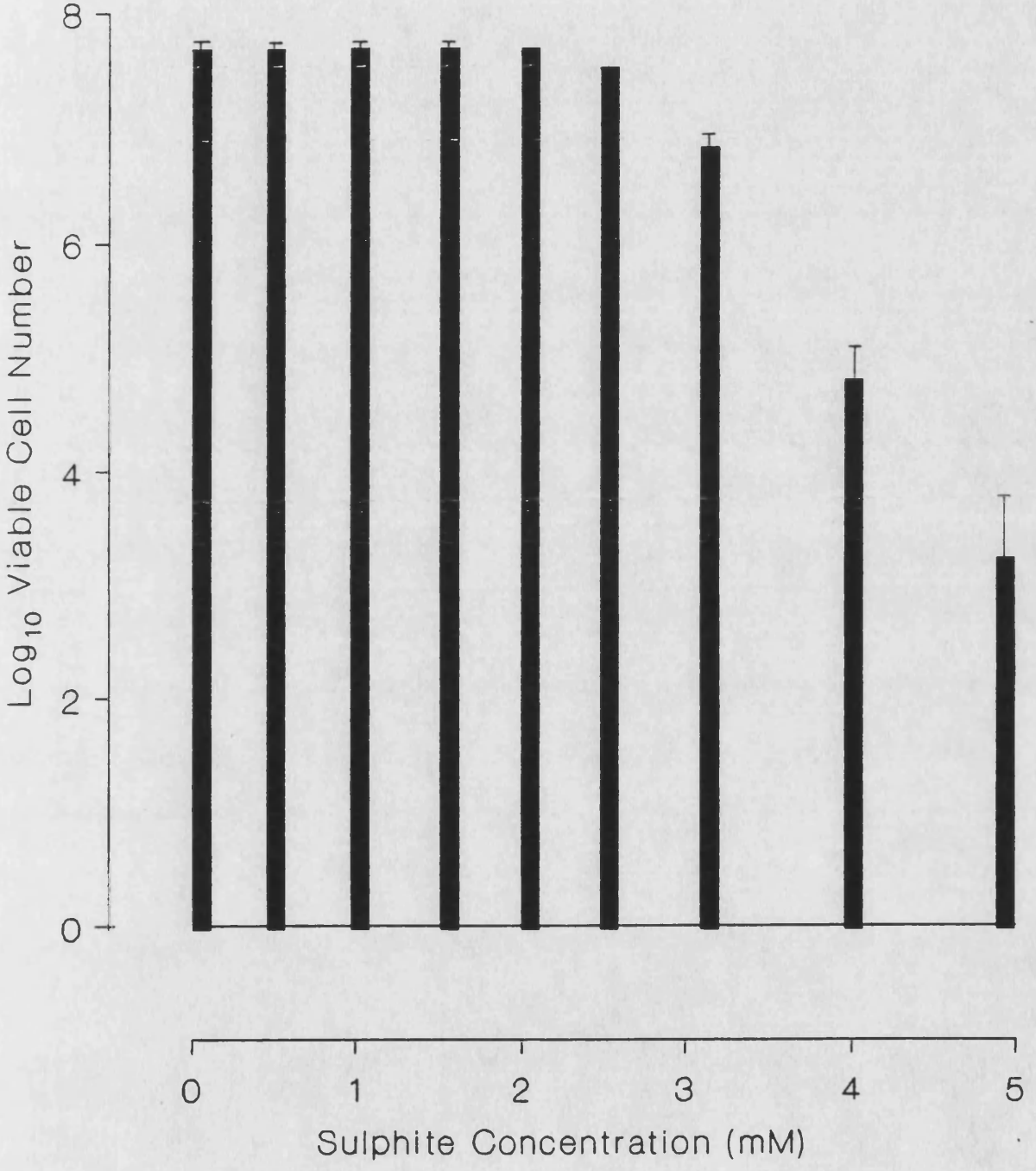
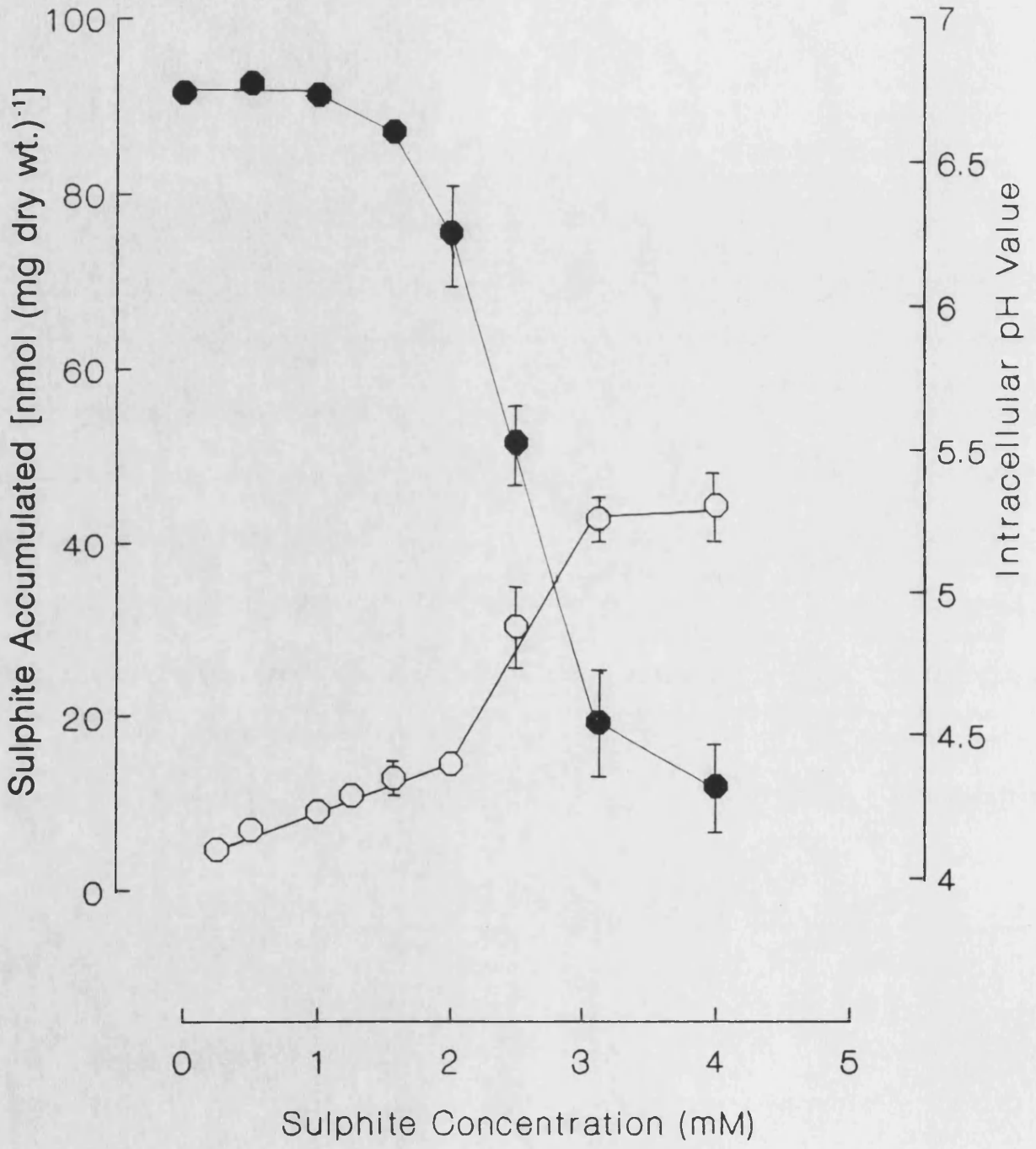
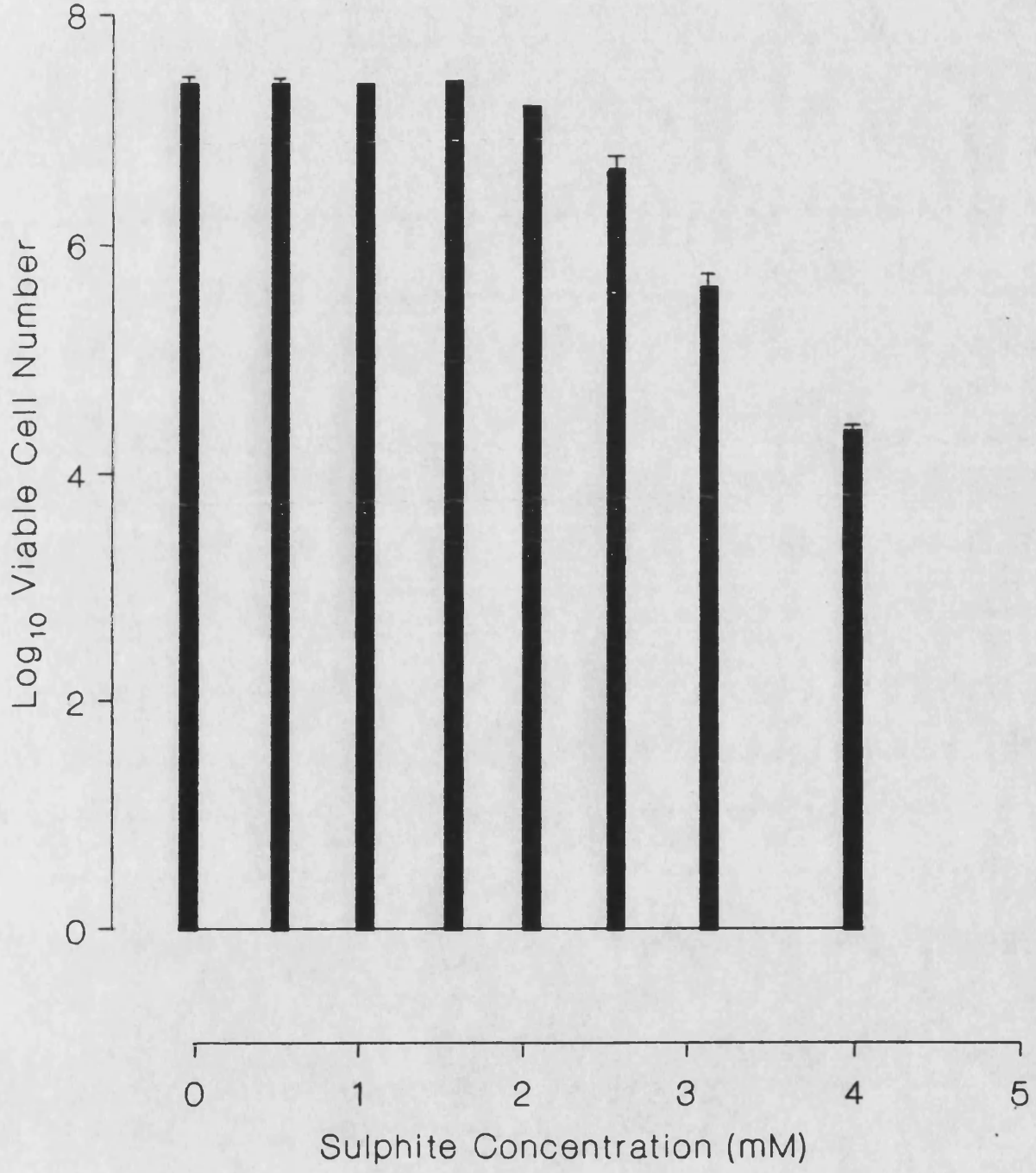

8
6
4
2
0
Log10 Viable Cell Number
0
1
2
3
4
5
Sulphite Concentration (mM)

Figure 14c(i)


Sulphite Accumulated [nmol (mg dry wt.)⁻¹]
100
80
60
40
20
0
7
6.5
6
5.5
5
4.5
4
Intracellular pH Value
0
1
2
3
4
5
Sulphite Concentration (mM)

14c(ii)


8
6
4
2
0
Log10 Viable Cell Number
0
1
2
3
4
5
Sulphite Concentration (mM)

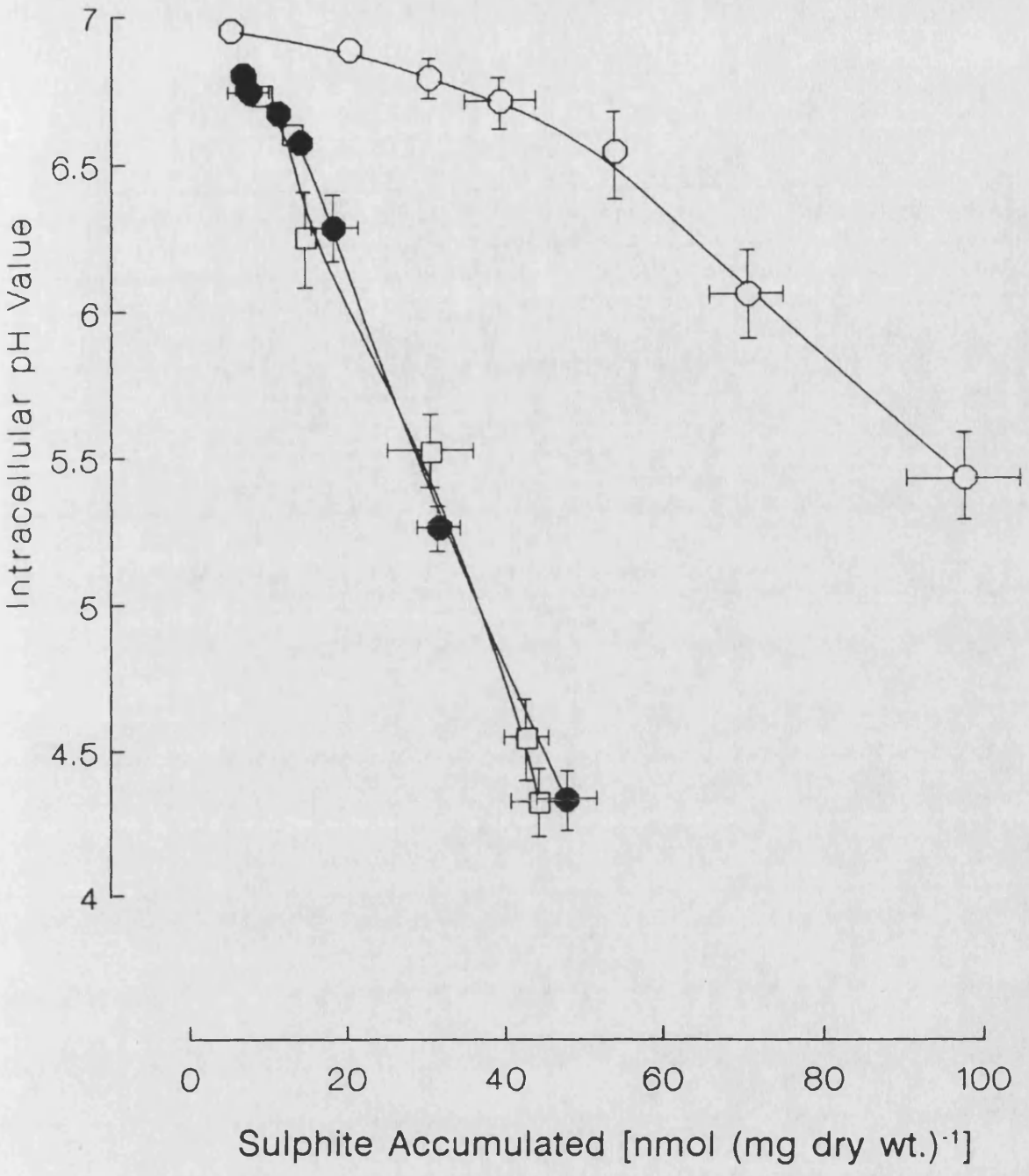


Figure 15. Intracellular buffering capacities of *Saccharomyces cerevisiae* AWRI 1A65 (○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□). Measurements were made after organisms (1 mg dry wt. ml^{-1}) had been suspended for 10 min in citric acid buffer (pH 4.0) containing glucose (100 mM) and either radioactively-labelled sulphite (0 - 5 mM; 0.2 - 0.6 μCi ml^{-1}) or non-radioactive sulphite (0 - 5 mM) supplemented with radioactively labelled propionic acid (2 μM; 0.5 μCi ml^{-1}). Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

accumulated most sulphite and, interestingly, showed the smallest decline in intracellular pH value. With both strains of *S'codes ludwigii*, the decline in intracellular pH value reflected the biphasic nature of sulphite accumulation. During the first phase of accumulation, where relatively small concentrations of sulphite entered organisms, intracellular pH values declined slowly; after the onset of the second phase, where relatively large concentrations of sulphite were accumulated, intracellular pH values decreased markedly. The declines in intracellular pH values, with all three strains, were described by sigmoid curves, the lower ranges of which corresponded to sulphite-saturation of organisms. The mid-range of these curves could be associated with a decrease in viability of organisms.

By plotting intracellular pH values against sulphite accumulation, a relative measure of intracellular buffering capacity was obtained (Fig. 15). The intracellular buffering capacities of *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10 were found to be virtually identical, and were substantially smaller than the intracellular buffering capacity of *Sacch. cerevisiae* AWRI 1A65.

APPARENT INTRACELLULAR pH VALUES CALCULATED FROM SULPHITE-UPTAKE DATA.

A model system for the interaction of sulphite with yeast cells was devised on the basis of the following assumptions:

(a) Intracellular and extracellular environments were separated by a single barrier, the plasma membrane, and only molecular sulphur dioxide could traverse this barrier.

(b) At equilibrium, concentrations of molecular sulphur dioxide were the same in the intracellular and extracellular environments.

(c) Concentrations of molecular sulphur dioxide could be accurately calculated as proportions of total sulphite by assuming pK_a values of 1.77 and 7.20 for the first and second dissociations of sulphur dioxide.

(d) Binding of sulphite in the intracellular and extracellular environments or potential anion leakage from cells would not be taken into account.

Intracellular pH (pHi) values were calculated using the following expression:

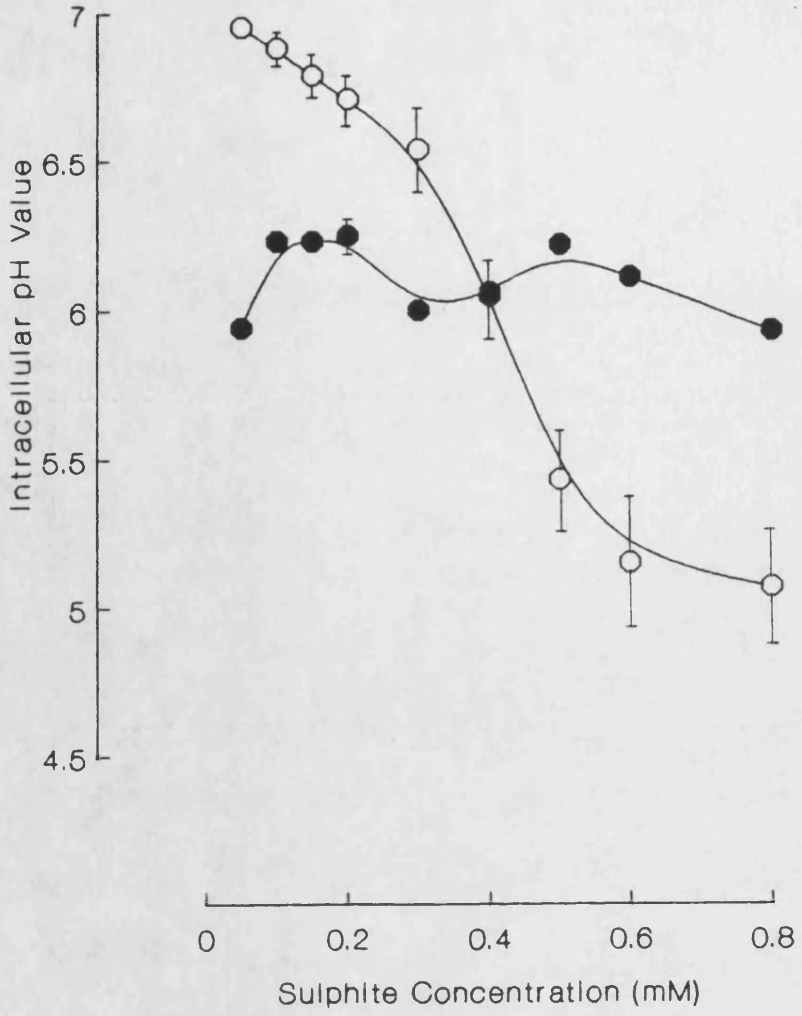
$$pHi = \log_{10} \left[\frac{100}{P} \frac{Ti\ Ve}{Te\ Vi}\right] + pK_1$$

(Appendix 2). The variables Ti and Te are the intracellular and extracellular total concentrations of sulphite, Vi and Ve are the intracellular and extracellular volumes, pK_1 is the dissociation constant for the reaction leading to the ionization of sulphur dioxide, and P is the percentage of extracellular sulphite which is in the form of molecular sulphur dioxide; at pH 4.0, P = 0.585 %. Intracellular pH values calculated in this manner were not in accordance with those measured using radioactively-labelled propionic acid (Fig. 16). However, general trends did not change; *Sacch. cerevisiae* AWRI 1A65 was found to possess both a higher intracellular pH value and a larger intracellular buffering capacity than either strain of *S'codes ludwigii* (Fig. 17). The significance of these findings will be addressed in the Discussion section.

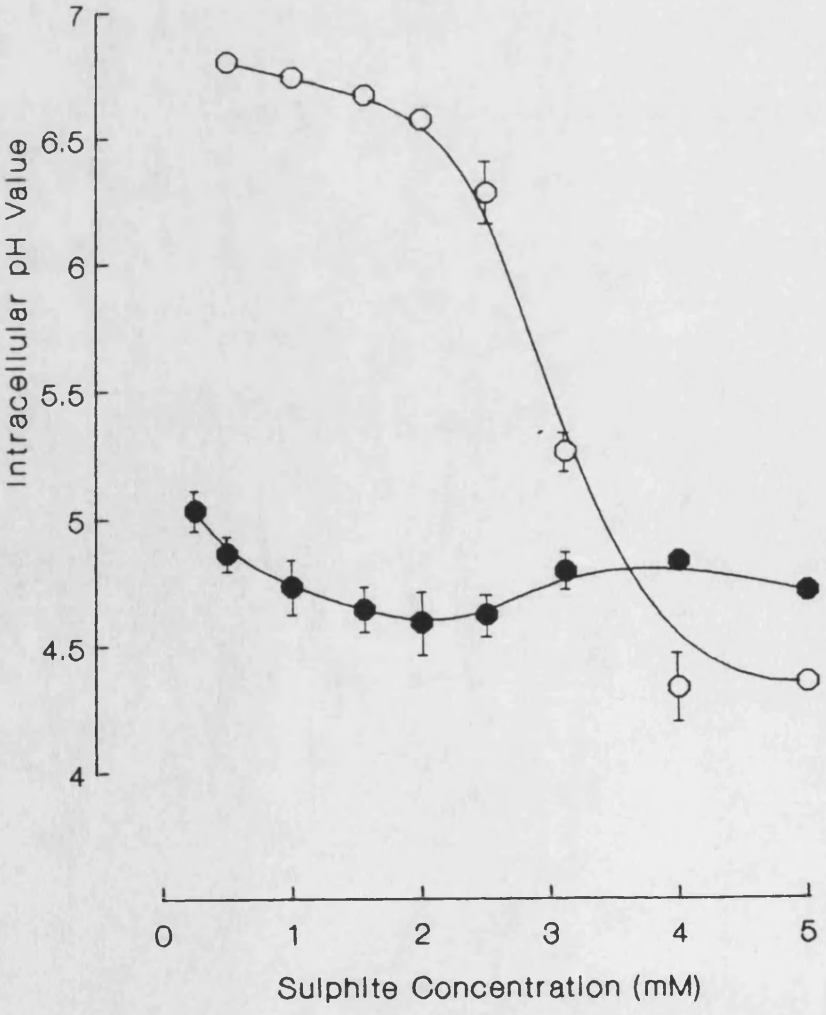
EFFECTS OF SULPHITE ON INTRACELLULAR pH VALUES WITH RESPECT TO CULTURE AGE.

Accumulation of sulphite by all three yeast strains had least effect on intracellular pH values of early-exponential phase organisms, and greatest effect on intracellular pH values of mid-exponential phase organisms (Fig. 18). The declines in intracellular pH values of *Sacch. cerevisiae* AWRI 1A65, in all phases of growth,

Figure 16a



16b



16c

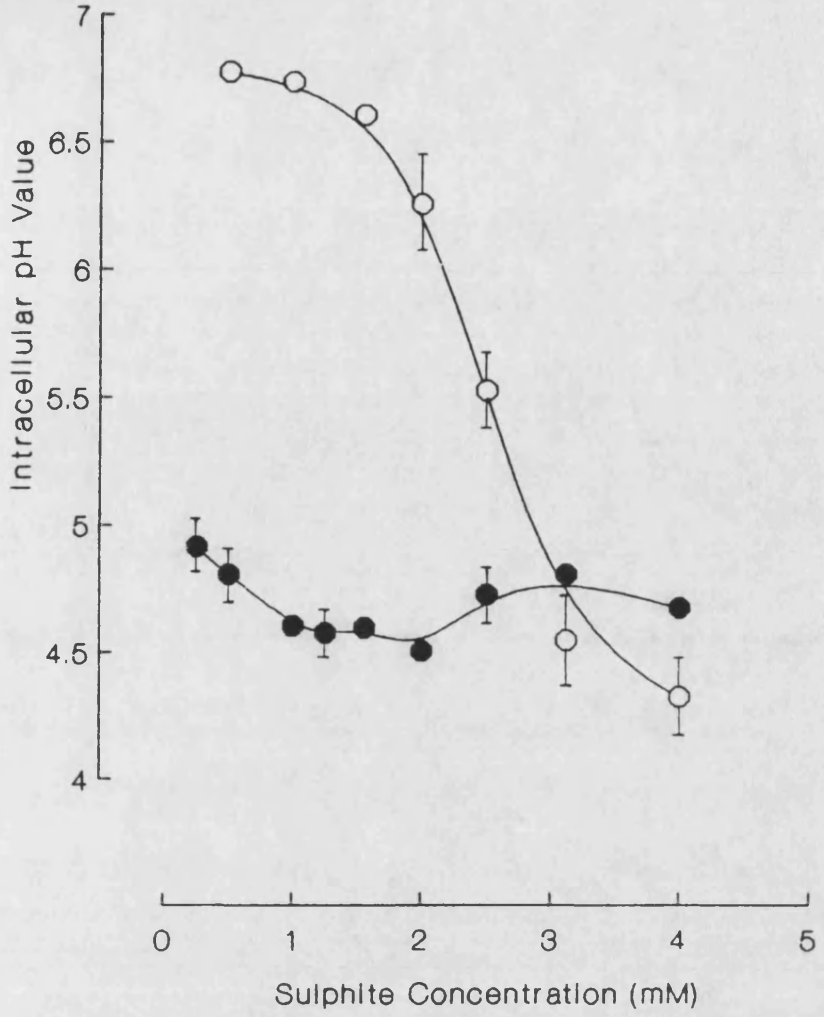


Figure 16. Comparison of intracellular pH values measured using radioactively-labelled propionic acid (○) with those calculated from sulphite-uptake data (●) for *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 16a), *Saccharomycodes ludwigii* BC1 (Fig. 16b) and *Saccharomycodes ludwigii* TC10 (Fig. 16c). The calculation of intracellular pH values from sulphite-uptake data assumed pK_a values of 1.77 and 7.20, respectively, for the first and second dissociations of sulphur dioxide, and yeast intracellular volumes of 1.179, 1.725 and 2.291 μl (mg dry wt.)$^{-1}$ for *Saccharomyces cerevisiae* AWRI 1A65, *Saccharomycodes ludwigii* BC1 and *Saccharomycodes ludwigii* TC10, respectively. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

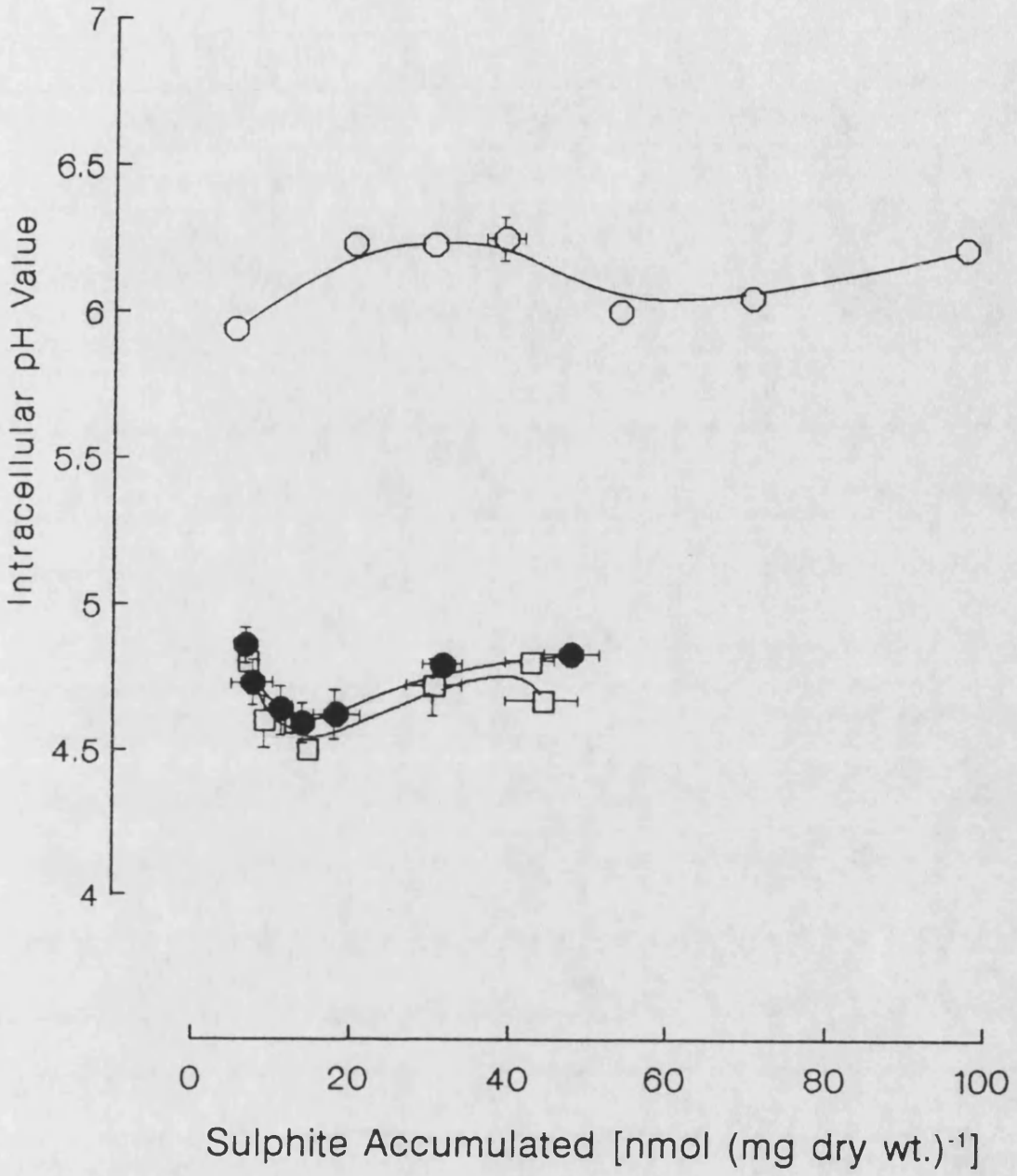
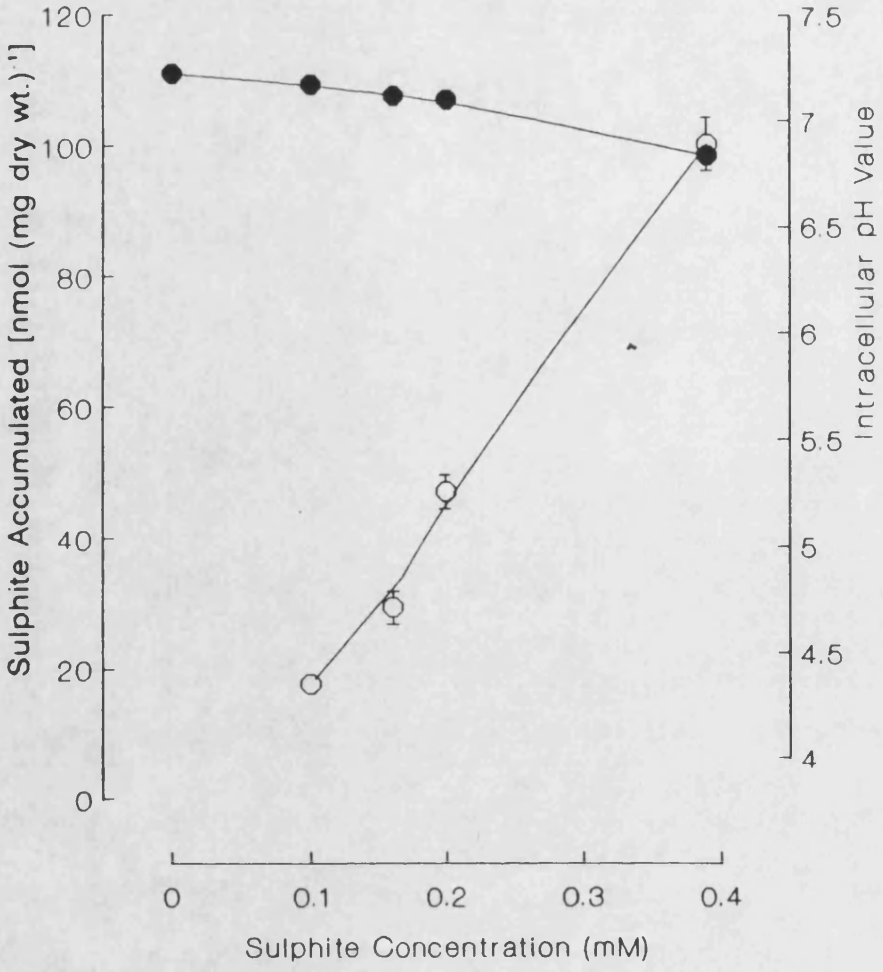
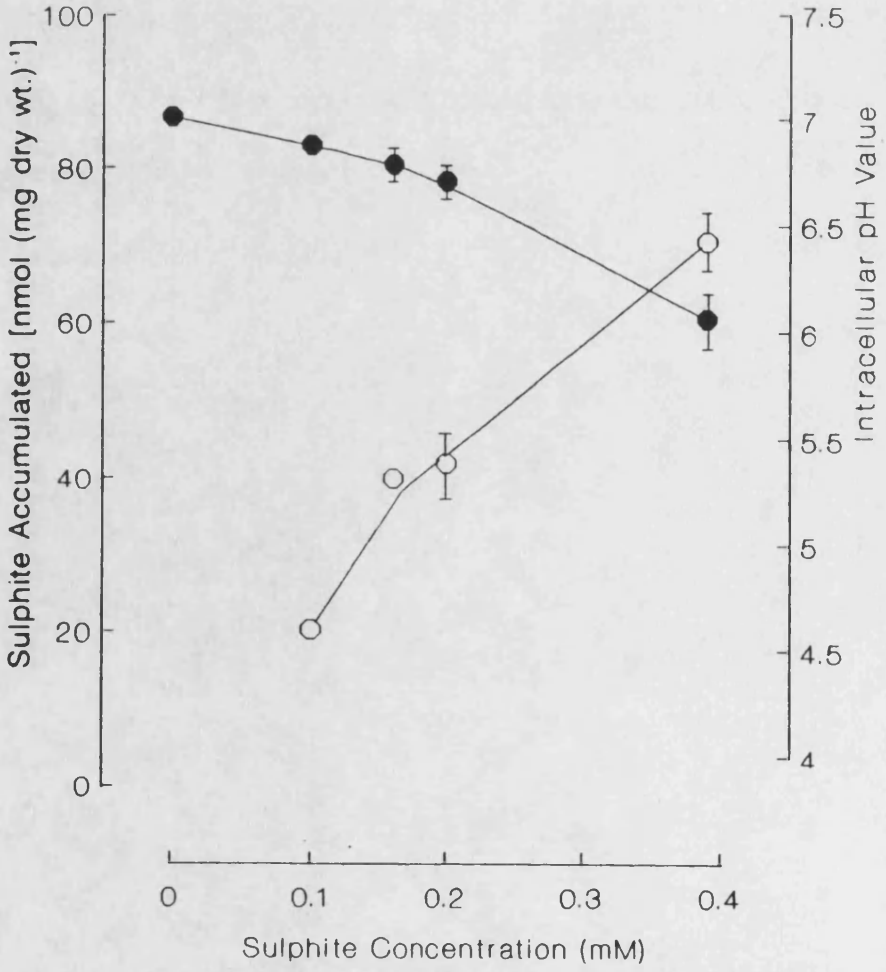


Figure 17. Relationship between accumulation of sulphite and intracellular pH values in *Saccharomyces cerevisiae* AWRI 1A65 (○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□). Intracellular pH values were calculated from sulphite-uptake data, as described for Figure 16. Bars indicate S.D., unless error lies within area of data point.

Figure 18a(i)



18a(ii)



18a(iii)

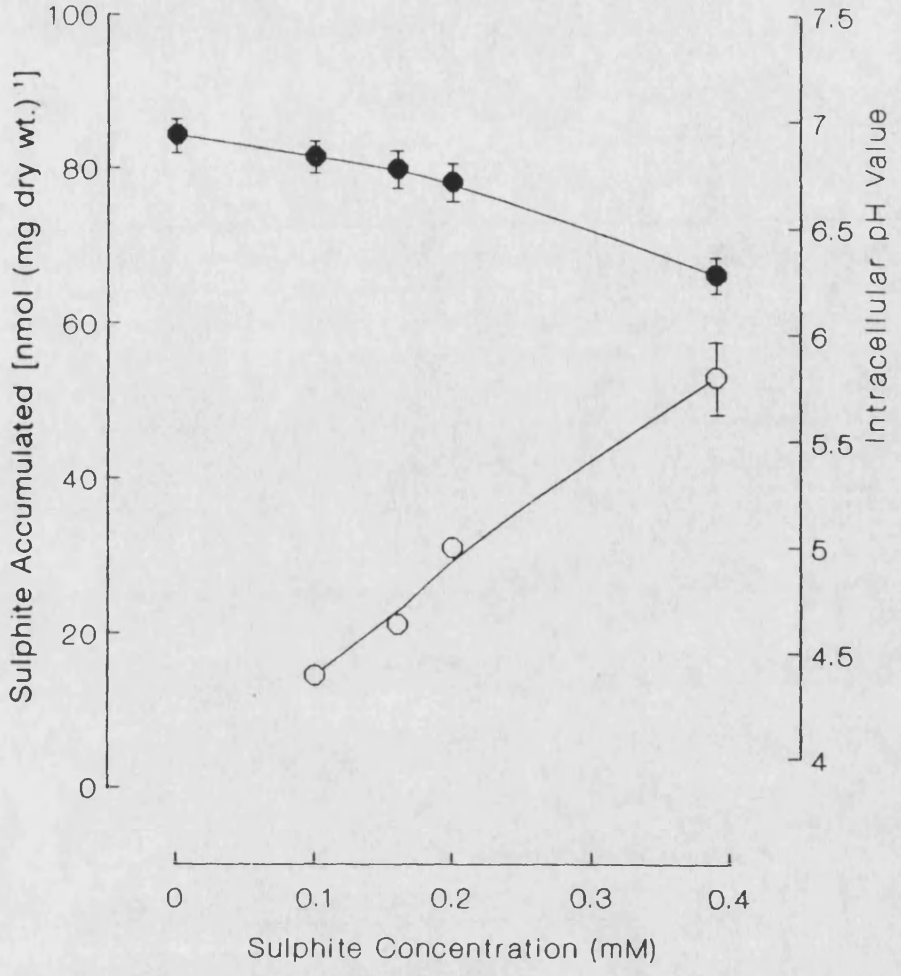
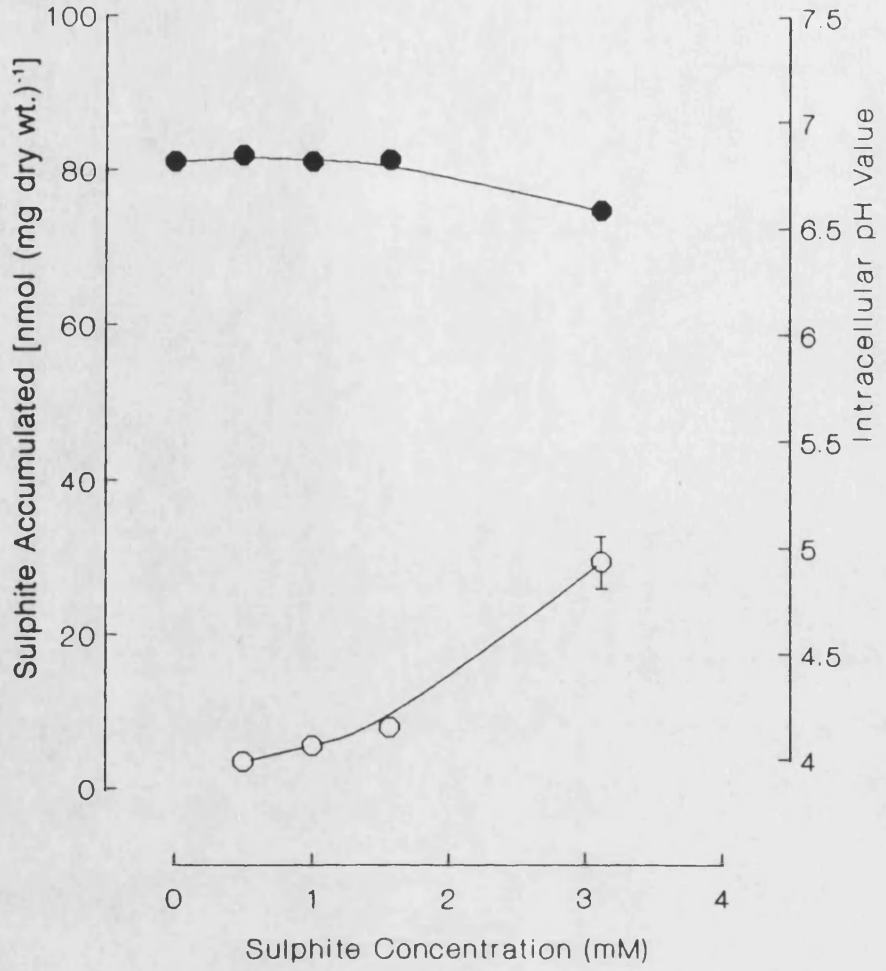
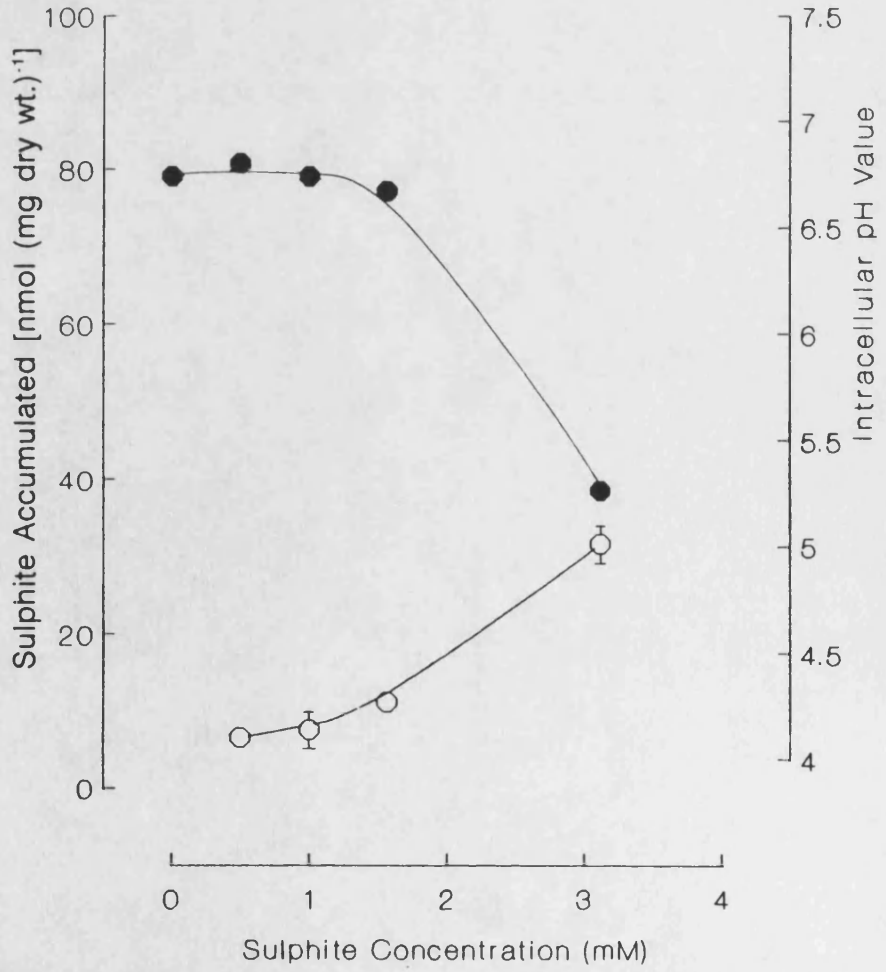


Figure 18a. Relationship between extent of accumulation of sulphite (○) and change in intracellular pH values (●) of *Saccharomyces cerevisiae* AWRI 1A65 with respect to culture age. Organisms were harvested at early- (i) or mid- (ii) exponential phases, or stationary (iii) phase of growth. Measurements were made after organisms (1 mg dry wt. ml^{-1}) had been suspended for 10 min in citric acid buffer (pH 4.0) containing glucose (100 mM) and either radioactively-labelled sulphite (0 - 5 mM; 0.2 - 0.6 μCi ml^{-1}) or non-radioactive sulphite (0 - 5 mM) supplemented with radioactively labelled propionic acid (2 μM; 0.5 μCi ml^{-1}). Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

Figure 18b(i)



18b(ii)



18b(iii)

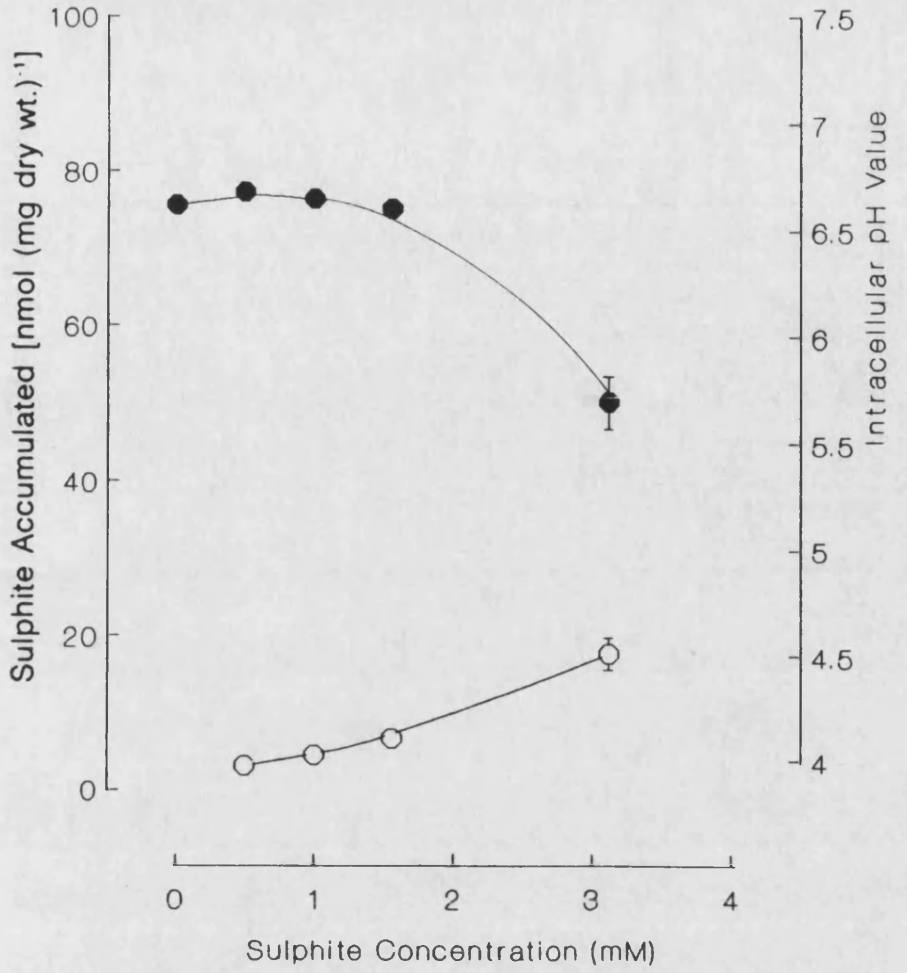
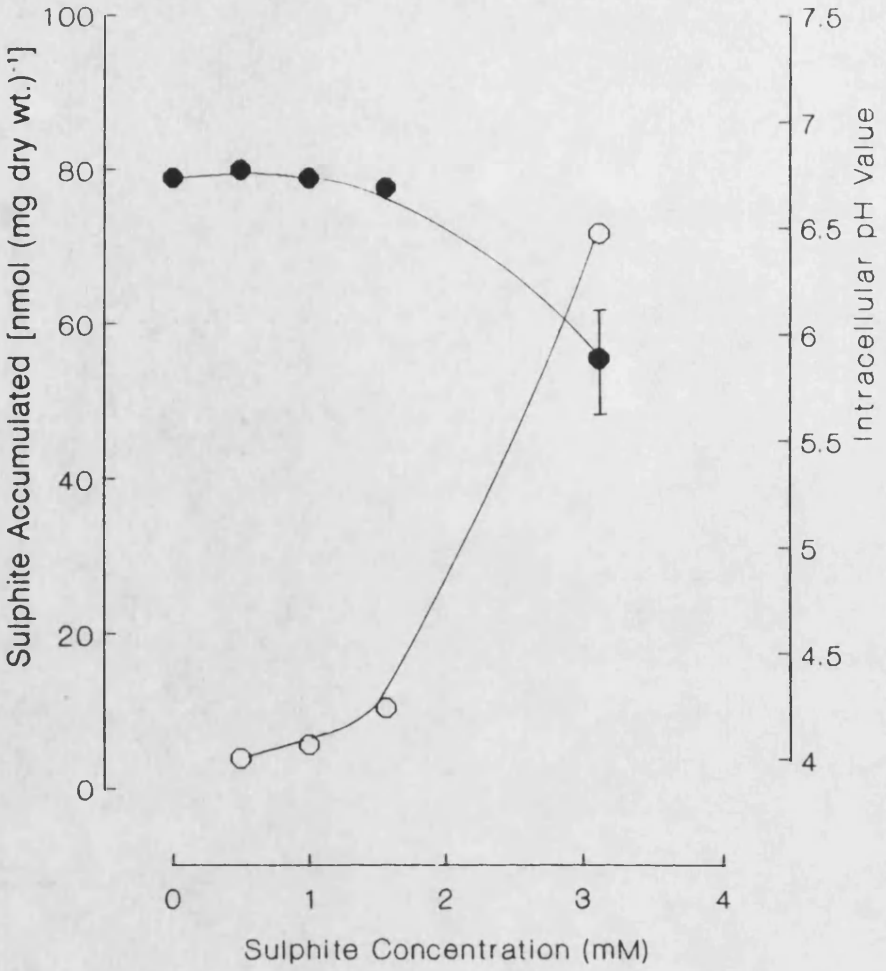


Figure 18b. Relationship between extent of accumulation of sulphite (○) and change in intracellular pH values (●) of *Saccharomycodes ludwigii* BC1 with respect to culture age. Organisms were harvested at early- (i) or mid- (ii) exponential phases, or stationary (iii) phase of growth. Measurements were made as described in Figure 18a. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

Figure 18c(i)



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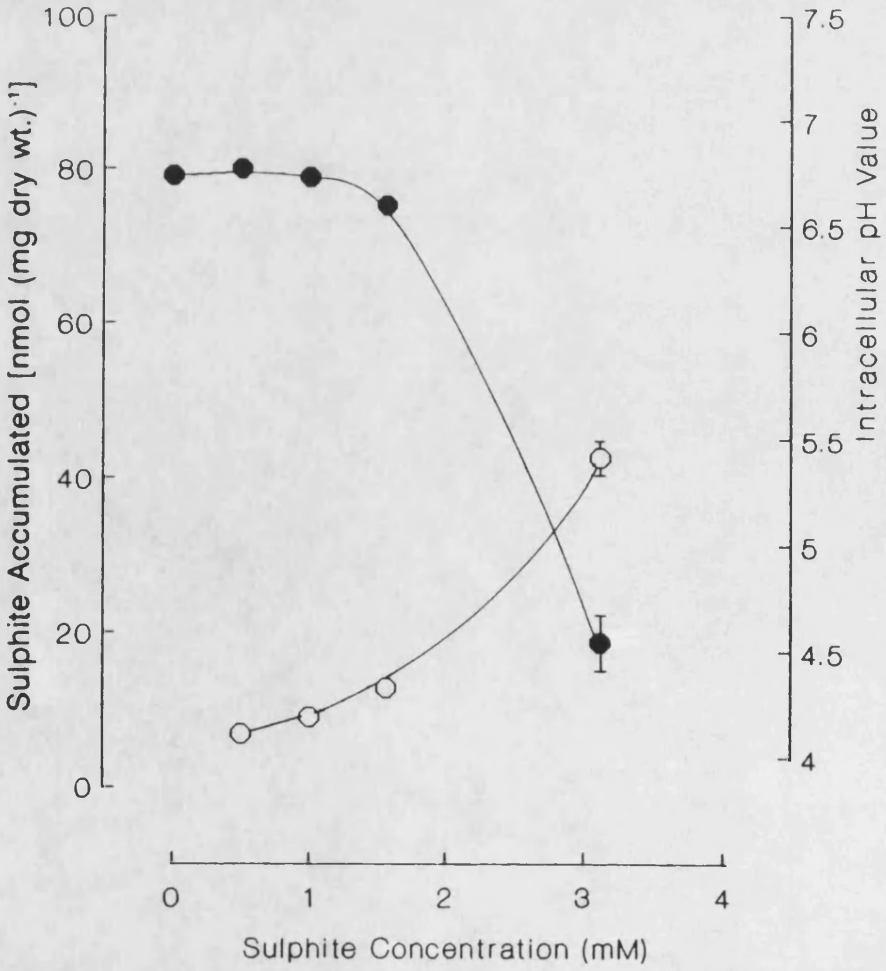


18c(iii)

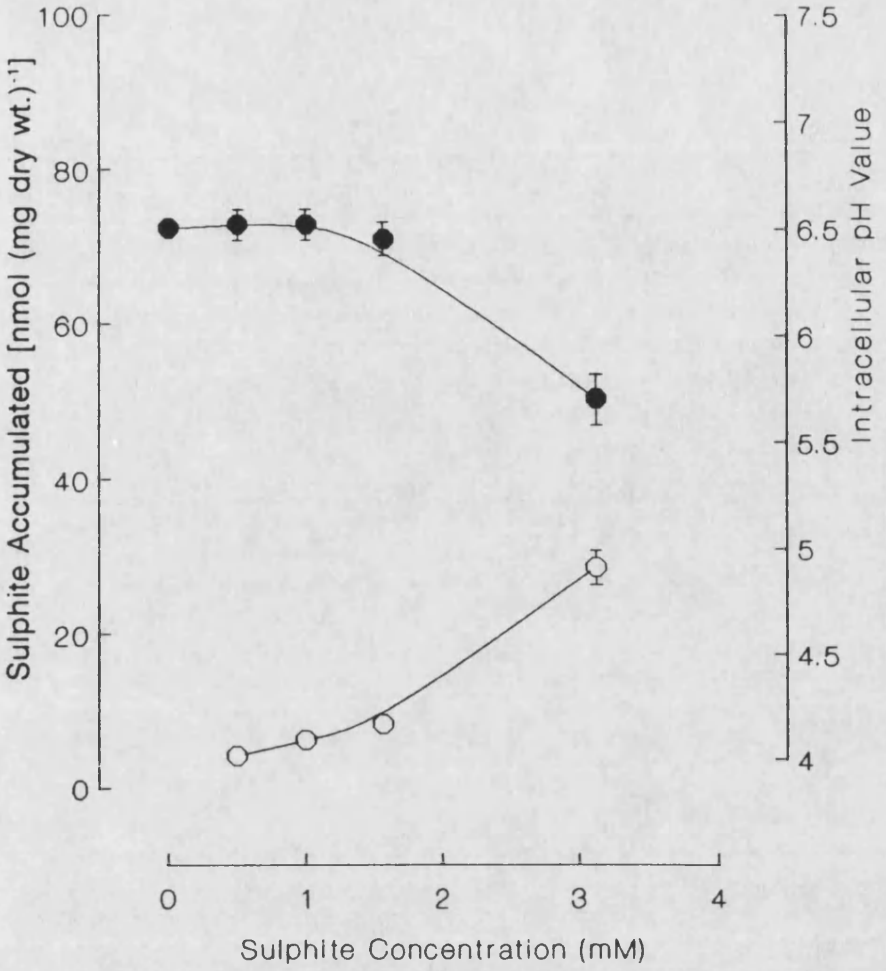
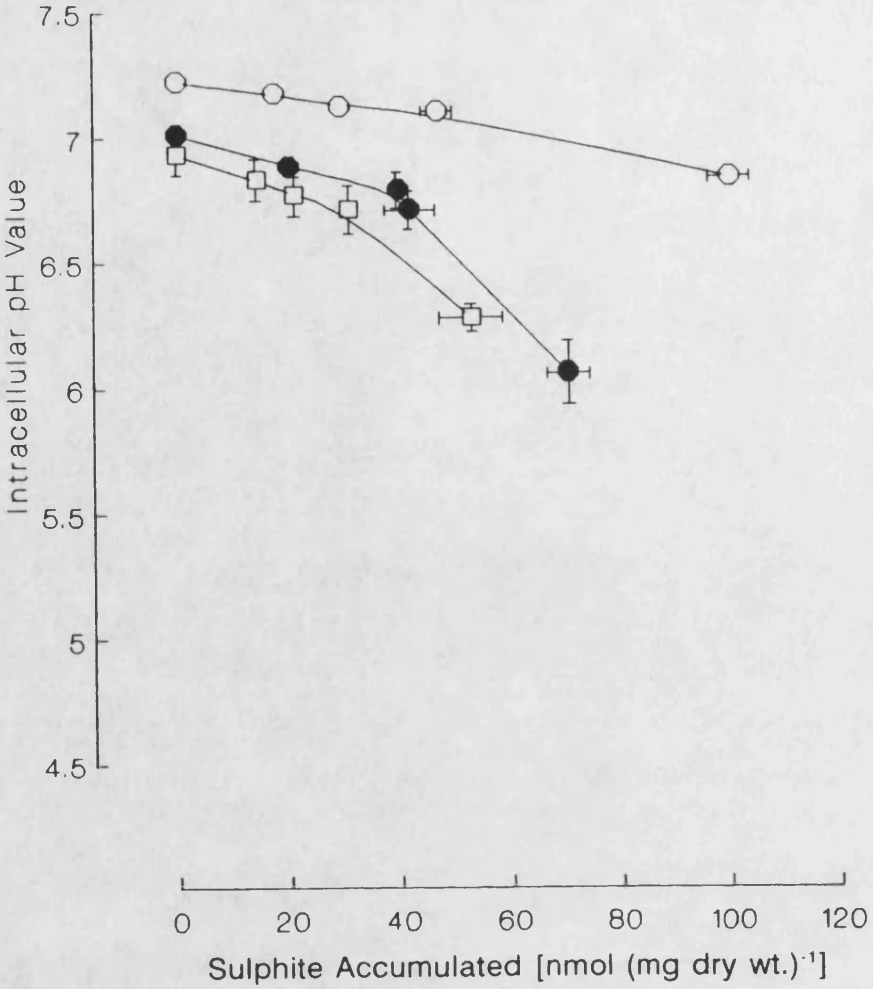
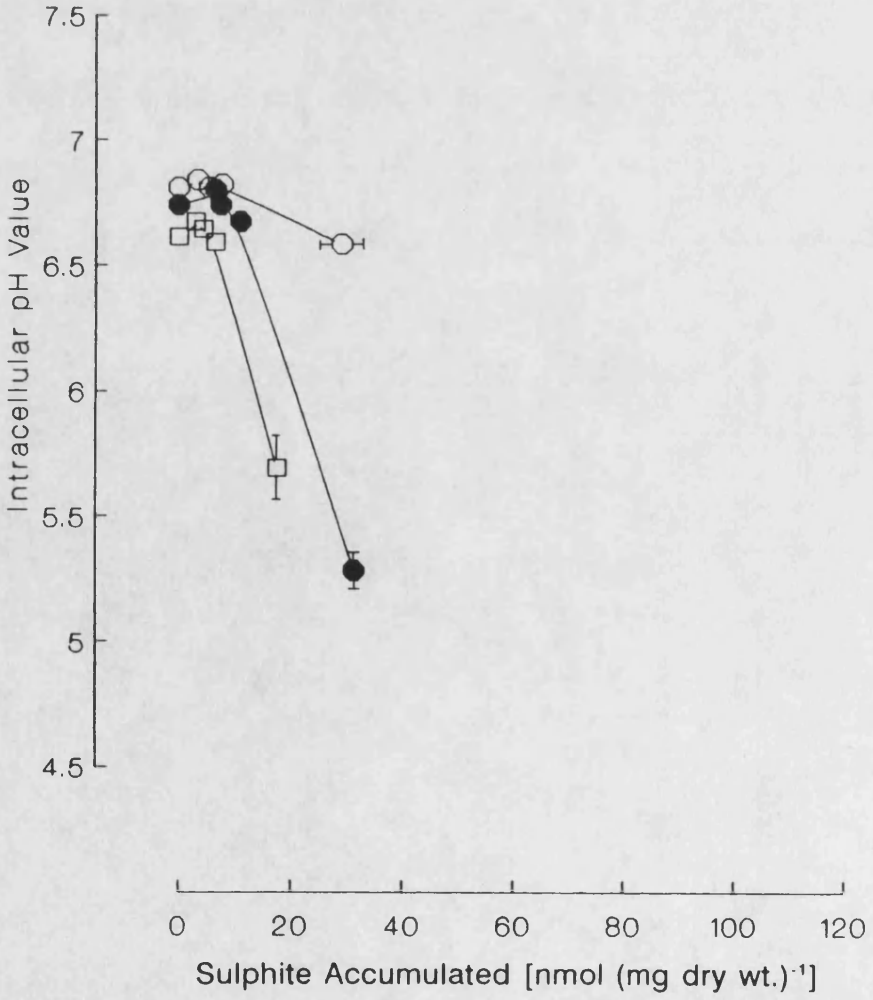


Figure 18c. Relationship between extent of accumulation of sulphite (○) and change in intracellular pH values (●) of *Saccharomycodes ludwigii* TC10 with respect to culture age. Organisms were harvested at early- (i) or mid- (ii) exponential phases, or stationary (iii) phase of growth. Measurements were made as described in Figure 18a. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

Figure 19a



19b



19c

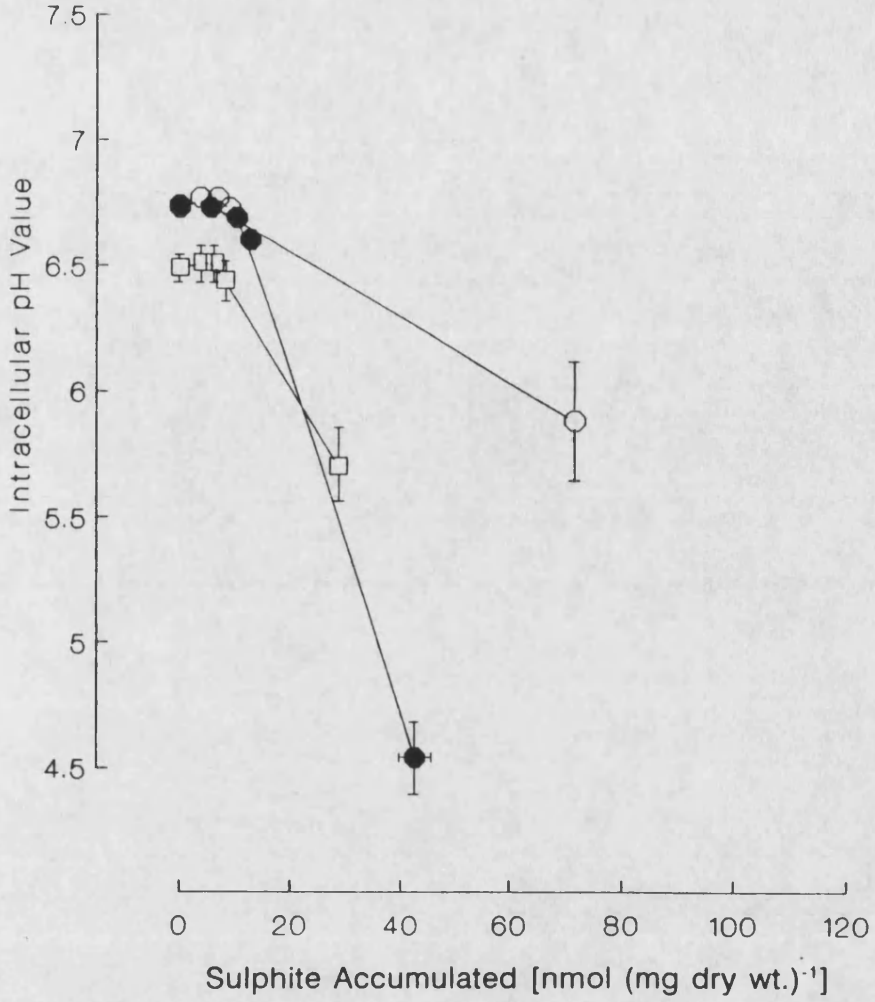
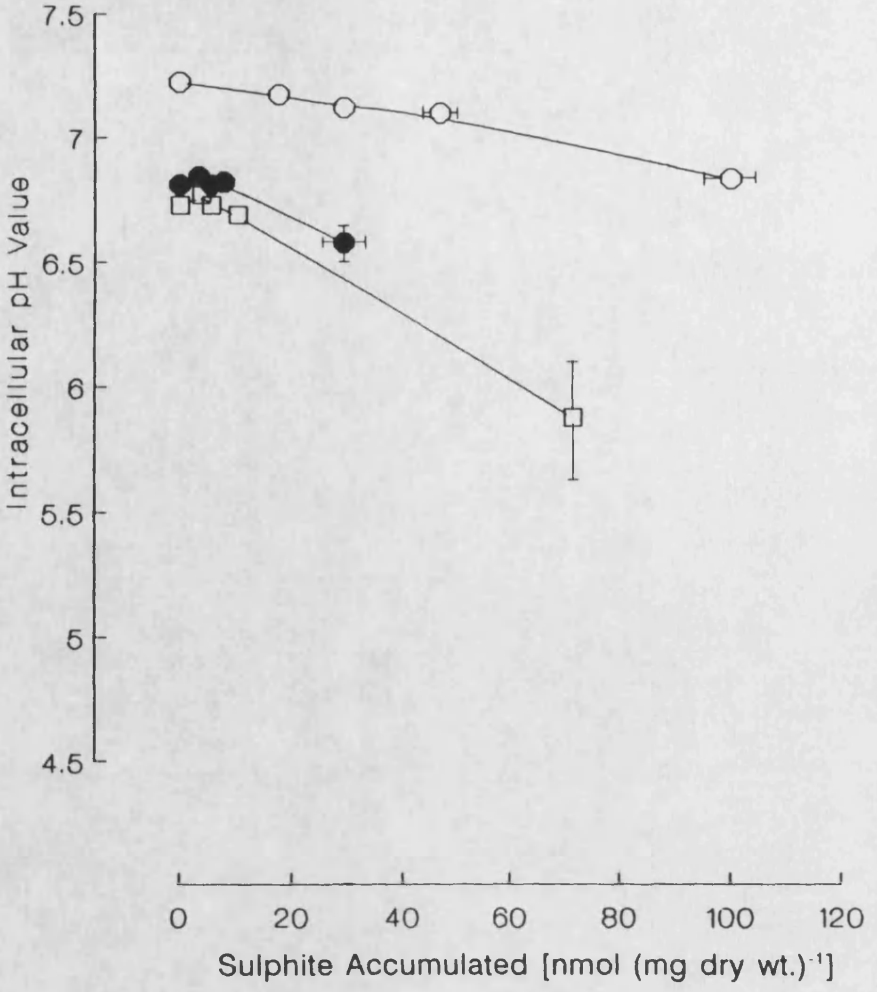
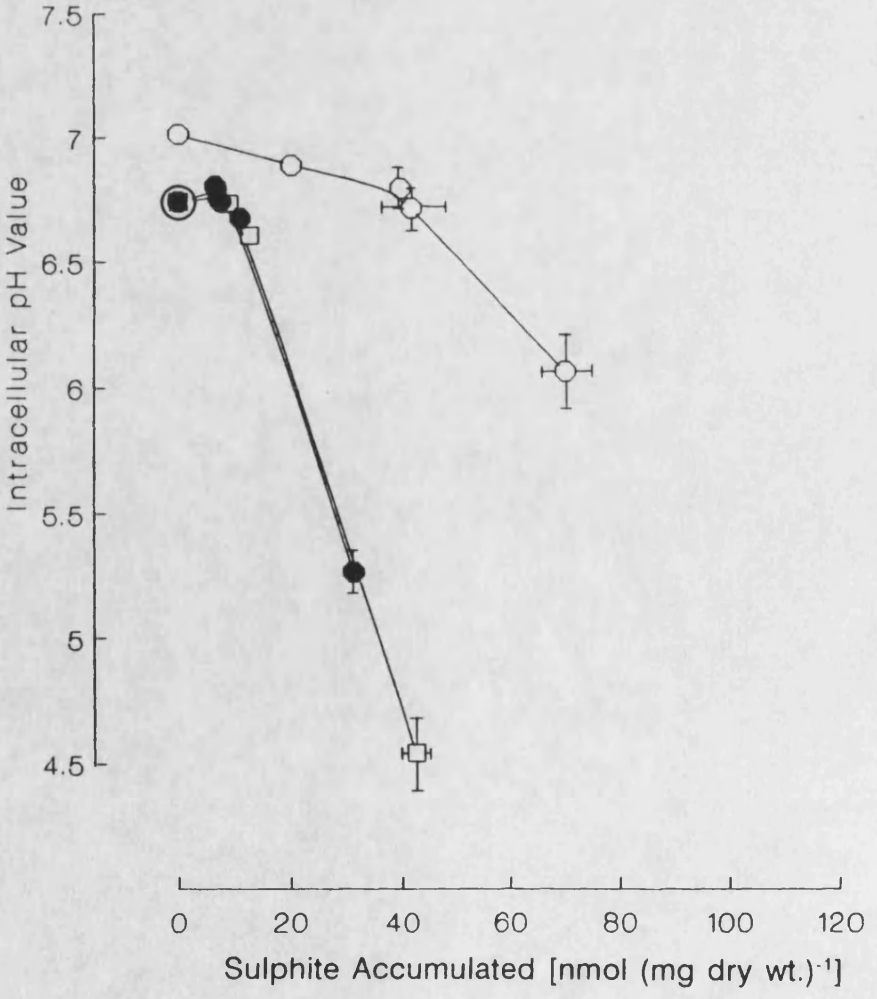


Figure 19. Intracellular buffering capacities of *Saccharomyces cerevisiae* AWRI 1A65 (Fig. 19a), *Saccharomycodes ludwigii* BC1 (Fig. 19b) and *Saccharomycodes ludwigii* TC10 (Fig. 19c) with respect to culture age. Organisms were harvested at early- (○) or mid- (●) exponential phases, or stationary (□) phase of growth. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

Figure 20a



20b



20c

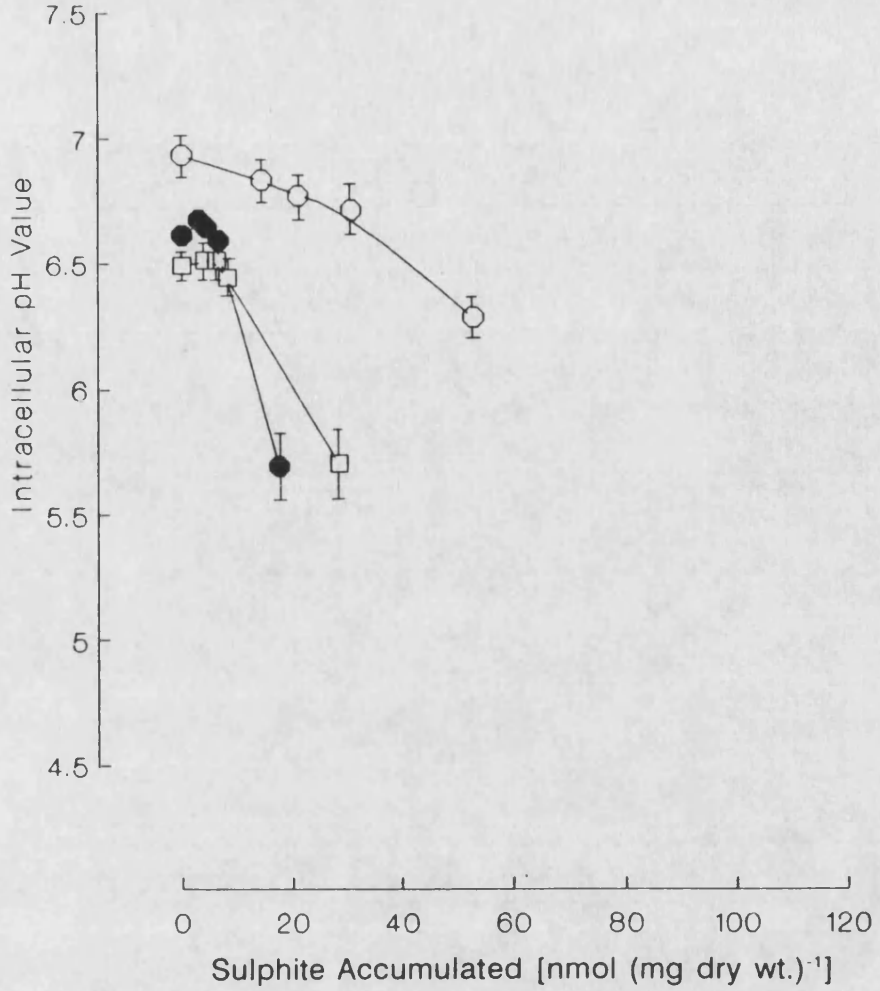


Figure 20. Intracellular buffering capacities of *Saccharomyces cerevisiae* AWRI 1A65 (○), *Saccharomycodes ludwigii* BC1 (●) and *Saccharomycodes ludwigii* TC10 (□) in different growth phases, with respect to each other. Organisms were harvested at early- (Fig. 20a) or mid- (Fig. 20b) exponential phases, or stationary (Fig. 20c) phase of growth. Values given are the means of at least three independent determinations. Bars indicate S.D., unless error lies within area of data point.

occurred gradually; with strains of *S'codes ludwigii*, intracellular pH values were maintained with constancy before a sharp drop. Intracellular buffering capacities, for *Sacch. cerevisiae* AWRI 1A65 and *S'codes ludwigii* BC1, were largest with cells from early-exponential phase and smallest in those from the stationary phase of growth (Fig. 19). The intracellular buffering capacity of *S'codes ludwigii* TC10 was also largest in cells from early exponential phase, but was lowest in those harvested from the mid-exponential phase of growth. Harvested in early- and mid-exponential phases and stationary phase, *Sacch. cerevisiae* AWRI 1A65 was observed to have larger intracellular buffering capacities than either strain of *S'codes ludwigii* (Fig. 20).

RESULTS (PART B)

EFFECTS OF SULPHITE ON FERMENTATIONS OF APPLE JUICE.

Fermentations of concentrate-derived apple-juice medium were considerably depressed by an initial concentration of 0.65 mM free sulphite, less so by 0.31 mM and enhanced by the presence of 0.10 mM free sulphite (Fig. 1). This enhancement was further evidenced by production of relatively high ethanol concentrations, and also by the fact that the most organoleptically acceptable ciders, in the opinion of an experienced tasting panel (results not shown), were those which originated from fermentations which had been supplemented with 0.78 mM total sulphite. Furthermore, these ciders originated from fermentations in which the pitching yeast, *Sacch. cerevisiae* AWRI 1A65, was dominant (Fig. 2). In fermentations of apple juice containing 2.34 mM total sulphite, *Sacch. cerevisiae* AWRI 1A65 was almost entirely undetectable, by the plate-count methods employed, for the duration of the experiment. The wild yeasts which dominated fermentations containing 2.34 mM total sulphite, and also those fermentations which were not pitched with *Sacch. cerevisiae* AWRI 1A65, were non-*Saccharomyces* species. They did not exhibit a greater sulphite tolerance than *Sacch. cerevisiae* AWRI 1A65, as shown by growth on agar media containing a range of sulphite concentrations. Their dominance in fermentations was associated with lower ethanol production and organoleptically unacceptable ciders.

The addition of wild yeast isolates to concentrate-derived apple juice, in order to increase the microbial load, resulted in all free sulphite becoming bound prior to pitching in every fermentation except those which had been supplemented with 2.34 mM total sulphite (Fig. 3). The free sulphite remaining was sufficient to suppress fermentation. Specific gravity decline was most rapid in fermentations which had been supplemented with 0.78 mM total sulphite, and this was reflected by the relatively high ethanol concentrations produced. However, fermentations

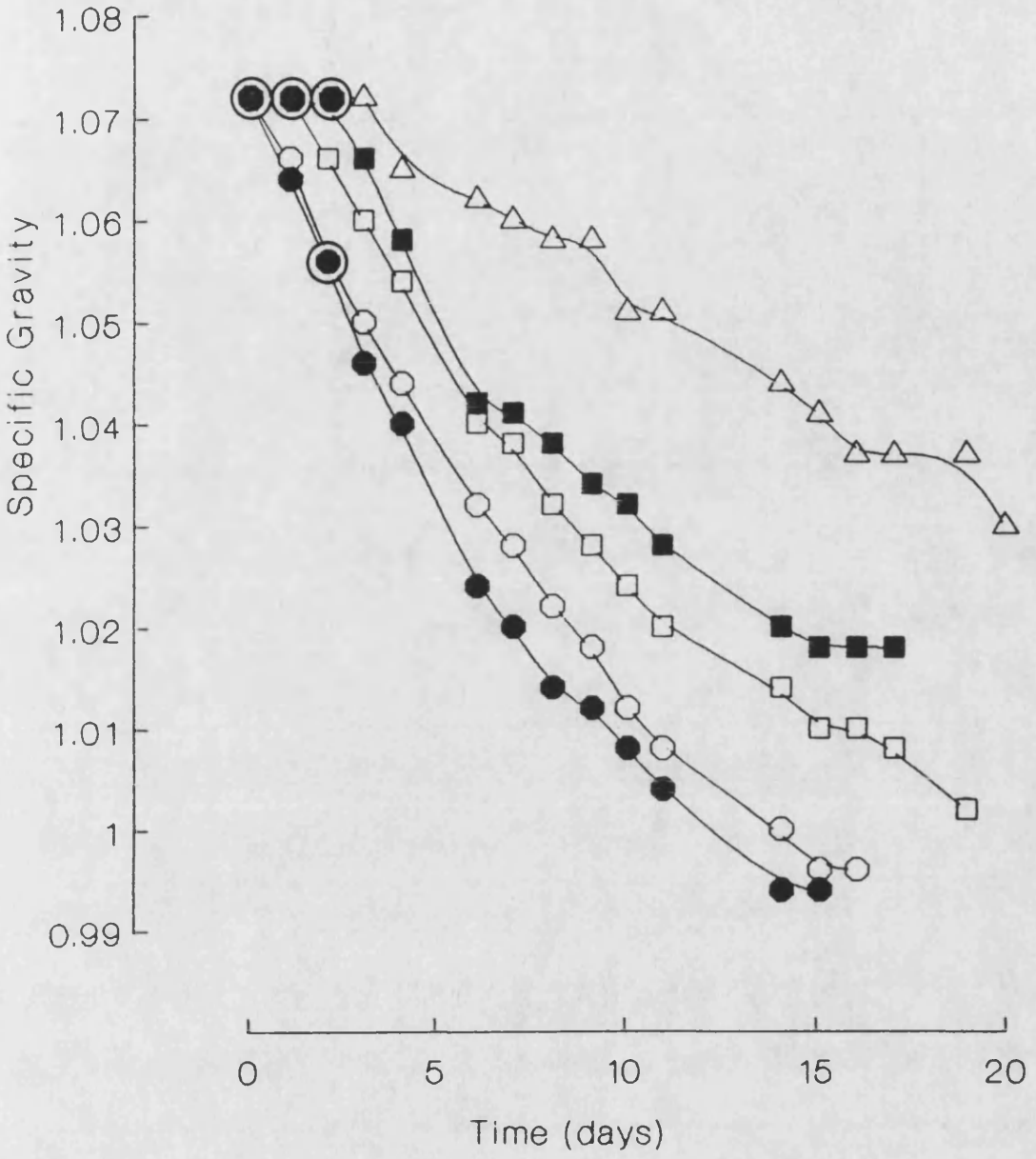
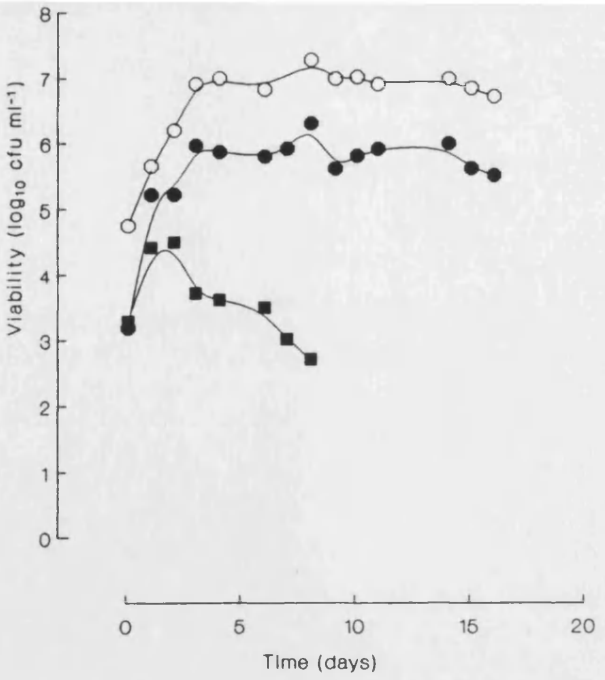
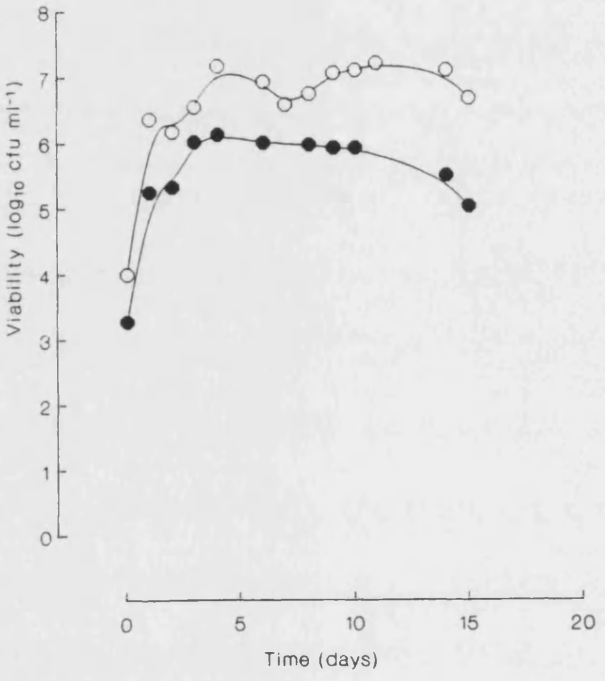


Figure 1. Time-course of change in specific gravity during fermentations of concentrate-derived apple-juice medium, either lacking sulphite (○) or containing 0.78 mM (●), 1.56 mM (□) or 2.34 mM (■ and △) total sulphite. All fermentations, except △, had been pitched with *Saccharomyces cerevisiae* AWRI 1A65 and incubated with stirring at 20°C. Initial free sulphite concentrations were, respectively, 0.00, 0.10, 0.31 and 0.65 mM. Final ethanol concentrations were, respectively, 5.1, 10.0, 10.0, 8.81 and 6.5 % (v/v). Results given are representative of at least two fermentations.

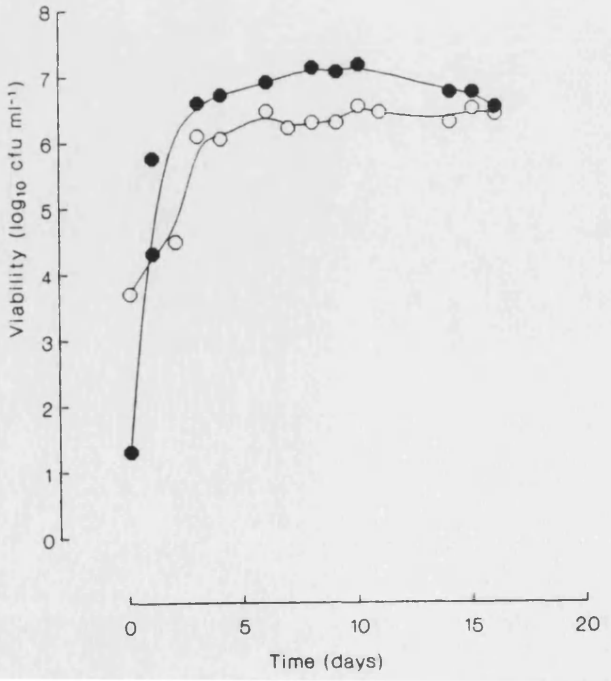
Figure 2a



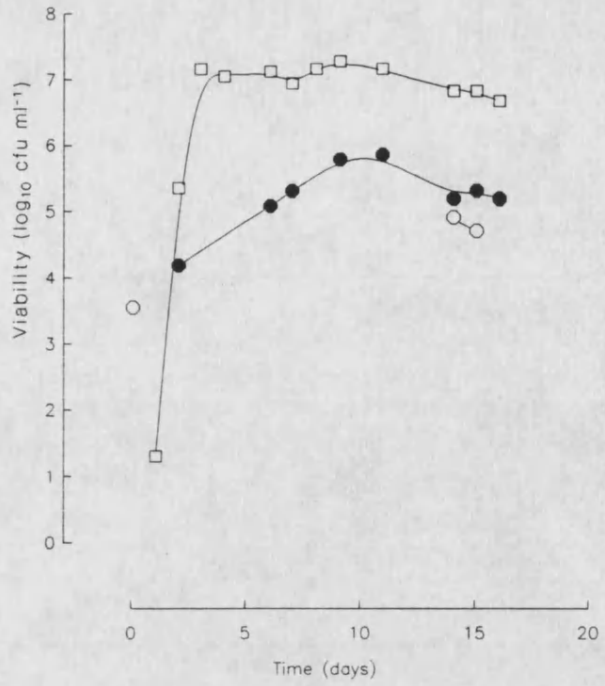
2b



2c



2d



2e

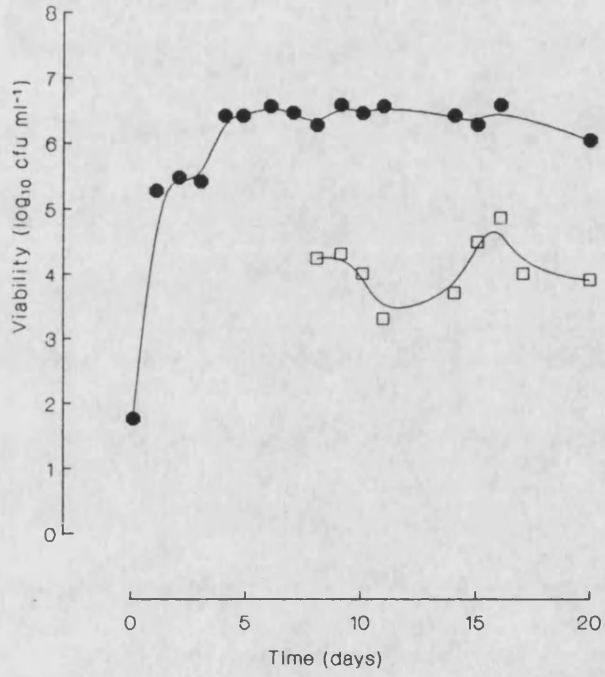


Figure 2. Time-course of change in viability of *Saccharomyces cerevisiae* AWRI 1A65 (◯) and three different wild yeasts (● , □ , ■) during fermentations of concentrate-derived apple-juice medium, either lacking sulphite (Fig. 2a) or containing 0.78 mM (Fig. 2b), 1.56 mM (Fig. 2c) or 2.34 mM (Fig. 2d and 2e) total sulphite. All fermentations, except Fig. 2e, had been pitched with *Saccharomyces cerevisiae* AWRI 1A65. Results shown pertain to Fig. 1, and are representative of at least two fermentations.

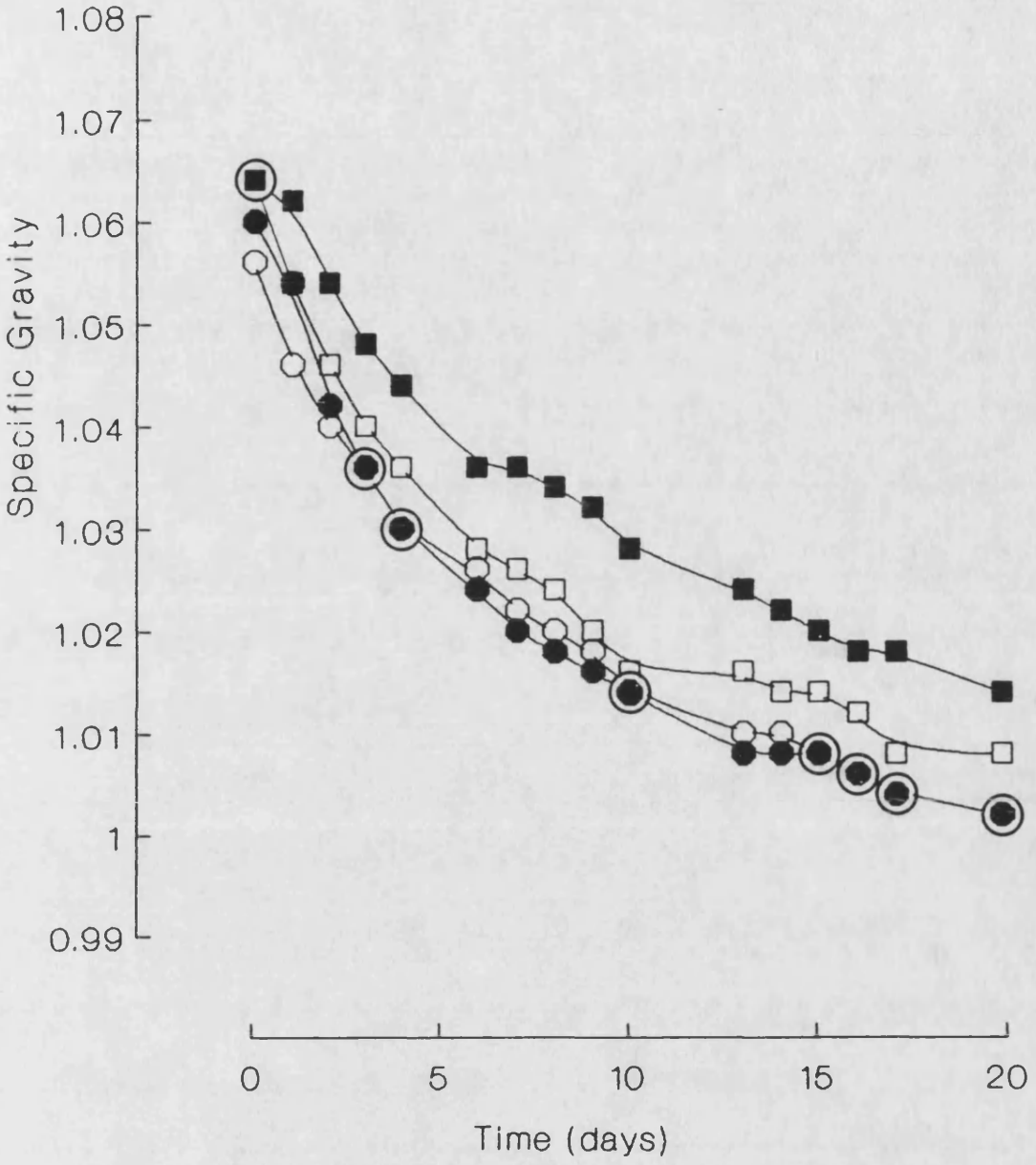
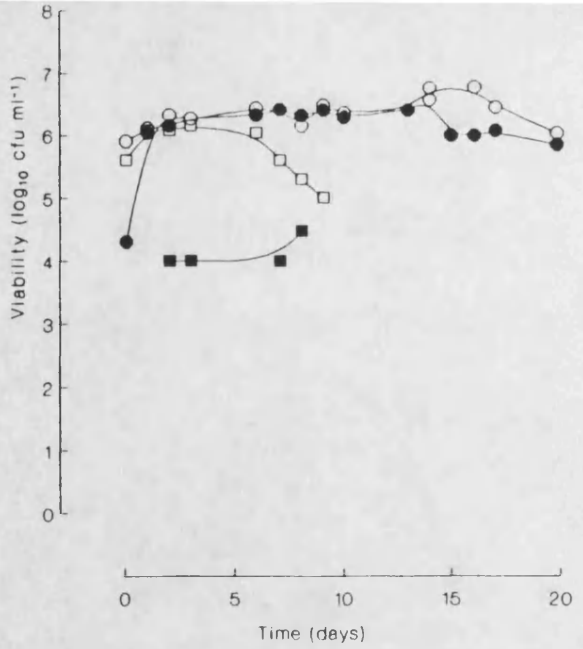
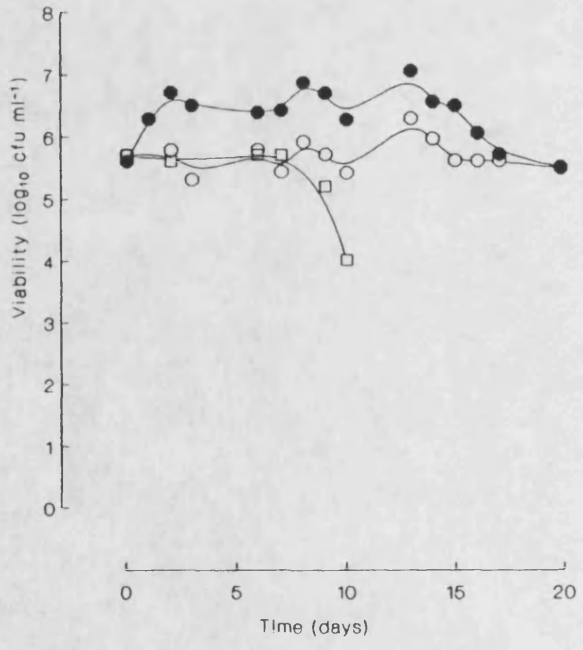


Figure 3. Time-course of change in specific gravity during fermentations of concentrate-derived apple-juice medium which had been inoculated with wild yeasts, isolated from fresh apple juice, and which either lacked sulphite (○) or contained 0.78 mM (●), 1.56 mM (□) or 2.34 mM (■) total sulphite. All fermentations had been pitched with *Saccharomyces cerevisiae* AWRI 1A65 and incubated with stirring at 20°C. Free sulphite was only detectable (0.23 mM) in ■ . Final ethanol concentrations were, respectively, 8.73, 9.15, 8.10 and 7.16 % (v/v). Results given are representative of at least two fermentations.

Figure 4a



4b



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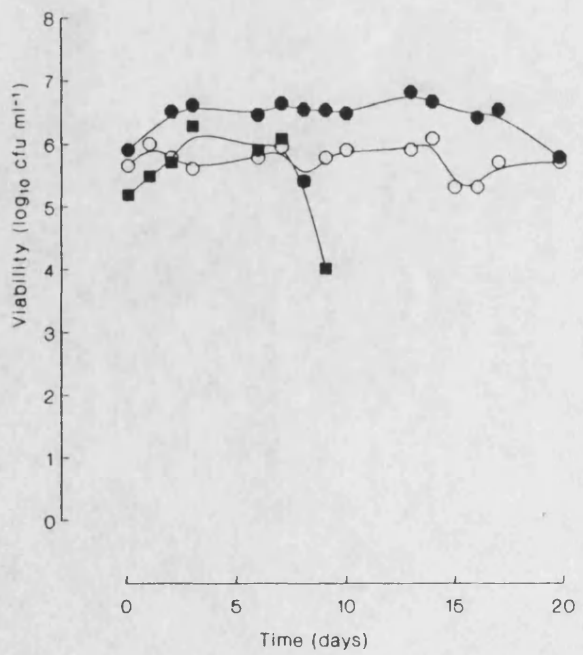


4d

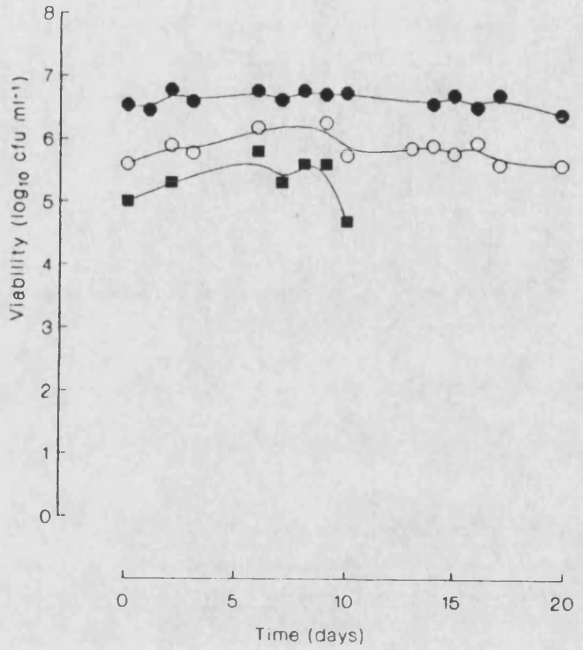


Figure 4. Time-course of change in viability of *Saccharomyces cerevisiae* AWRI 1A65 (○) and three different wild yeasts (● , □ , ■) during fermentations of concentrate-derived apple-juice medium which had been inoculated with wild yeasts, isolated from fresh apple juice, and which either lacked sulphite (Fig. 4a) or contained 0.78 mM (Fig. 4b), 1.56 mM (Fig. 4c) or 2.34 mM (Fig. 4d) total sulphite. Results shown pertain to Fig. 3, and are representative of at least two fermentations.

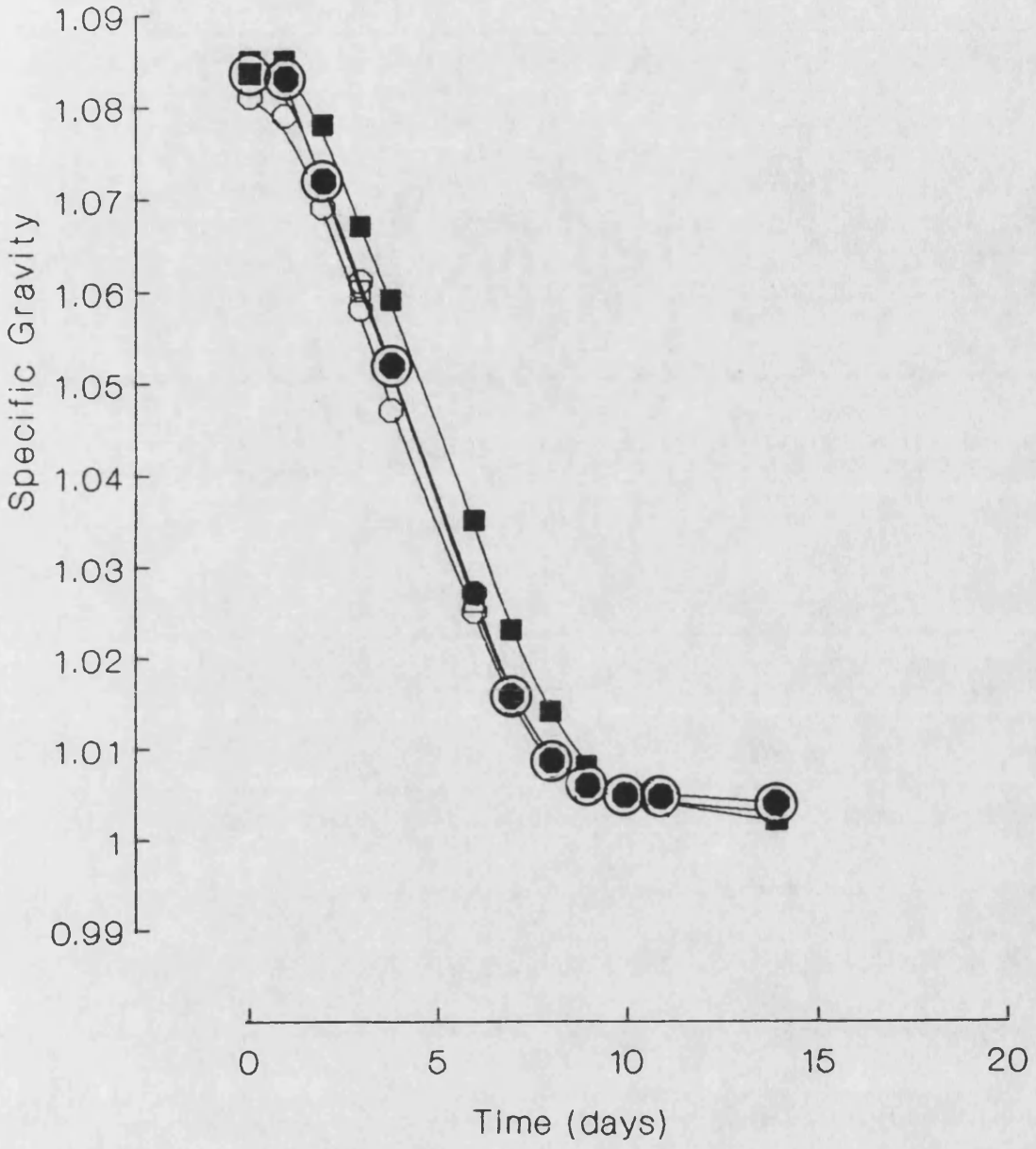
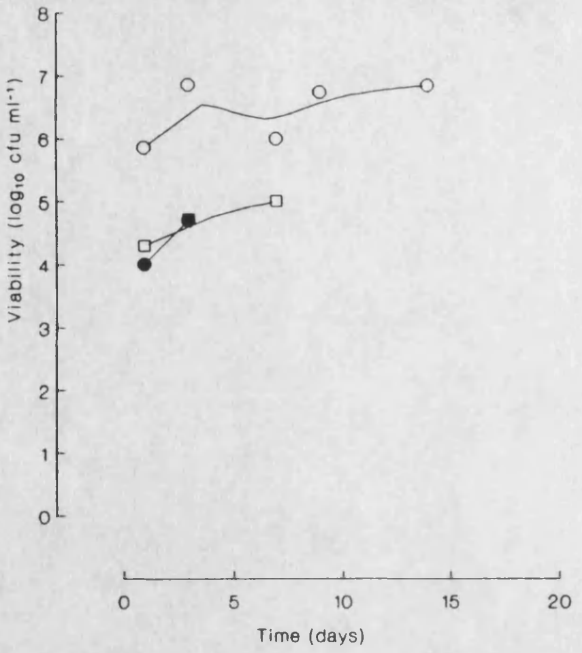
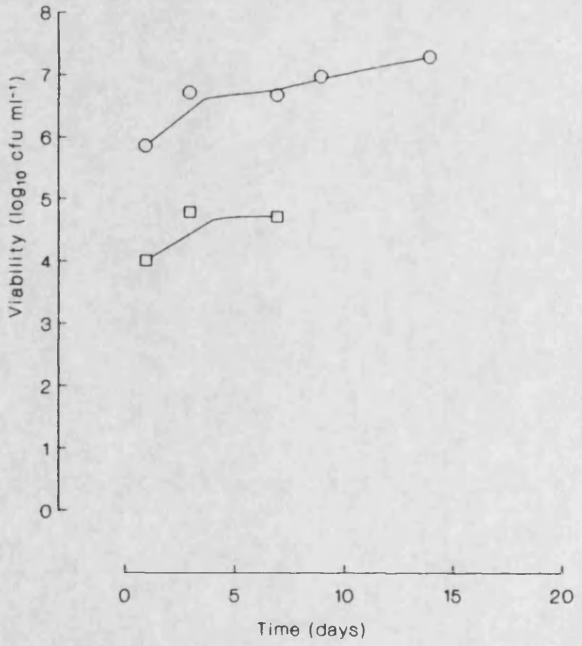


Figure 5. Time-course of change in specific gravity during fermentations of fresh apple-juice medium containing either 0.78 mM (○), 1.56 mM (●), 2.34 mM (□) or 3.12 mM (■) total sulphite. All fermentations had been pitched with *Saccharomyces cerevisiae* AWRI 1A65, and incubated with stirring at 20°C. Initial free sulphite concentrations were, respectively, 0.08, 0.10, 0.14 and 0.17 mM. Final ethanol concentrations were, respectively, 8.91, 9.45, 9.37 and 9.33 % (v/v). Results given are representative of at least two fermentations.

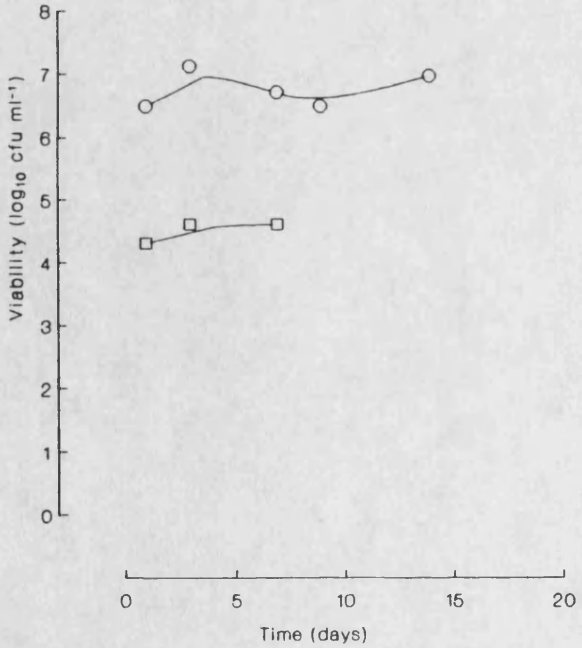
Figure 6a



6b



6c



6d

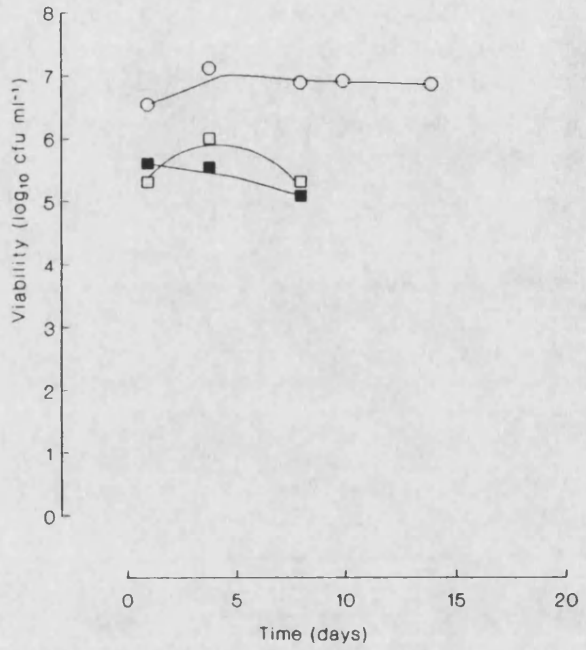


Figure 6. Time-course of change in viability of *Saccharomyces cerevisiae* AWRI 1A65 (○) and wild yeasts (● , □ , ■) during fermentations of fresh apple-juice medium containing either 0.78 mM (Fig. 6a), 1.56 mM (Fig. 6b), 2.34 mM (Fig. 6c) or 3.12 mM (Fig. 6d) total sulphite. Results shown pertain to Fig. 5, and are representative of at least two fermentations.

supplemented with 1.56 mM total sulphite resulted in the most organoleptically acceptable ciders (results not shown). There was no correlation between *Sacch. cerevisiae* AWRI 1A65 domination and production of superior ciders (Fig. 4). As with fermentations to which wild yeasts were not added, the yeasts which were prominent during these fermentations were non-*Saccharomyces* species, and did not demonstrate greater tolerances of sulphite than *Sacch. cerevisiae* AWRI 1A65.

The influence exerted by sulphite over fermentations of concentrate-derived apple juice was virtually absent from fermentations of fresh apple juice (Fig. 5). Decreased ethanol production, in fermentations supplemented with 3.12 mM total sulphite, could be associated with an excess of free sulphite present initially. *Saccharomyces cerevisiae* AWRI 1A65 domination was observed in all fermentations (Fig. 6), and all ciders produced were equally acceptable in terms of odour and taste.

Saccharomycodes ludwigii was not detected in any fermentation carried out either at Bath University or at HP Bulmer Technical Centre, Hereford. Furthermore, *S'codes ludwigii* was not present amongst a collection of 114 isolates obtained in a microbiological survey of the Hereford site (Autumn, 1989; results not shown). Similarly, this organism was not isolated from swabs taken from interiors of empty epoxide resin-lined tanks or oak vats (January 1991; results not shown).

DISCUSSION.

DISCUSSION

Sulphite Tolerances of Yeasts.

Saccharomyces cerevisiae AWRI 1A65 is a commercially available strain which is promoted by the Australian Wine Research Institute as a 'flocculating yeast which is used for preparing both red and white wine'. It is noted for producing significant amounts of higher alcohols and esters, giving the wine a floral character. *Saccharomyces cerevisiae* AWRI 1A65 is especially suited to growth in media containing low concentrations of nitrogenous nutrients, forms very compact lees, and is considered to be a low hydrogen sulphide-producer (P.A. Henschke, personal communication). It was surprising to find that a yeast which is used for wine-making, and now also cider-making, should be so intolerant of sulphite, since both processes are particularly dependent on the preservative. Prior to experimentation, it was not anticipated that viability of *Sacch. cerevisiae* AWRI 1A65 would be impaired at sulphite concentrations in the range 0.39 - 0.78 mM total sulphite, and rendered completely non-viable by higher concentrations. Stratford (1983) studied a similar system of sulphite tolerance with respect to cider-industry strains and found that his pitching yeast, *Sacch. cerevisiae* TC8, was more resistant to sulphite than the strain *Sacch. cerevisiae* NCYC 366; 1.2 and 0.5 mM total sulphite, respectively, were required to inhibit aerobic growth completely in media at 30°C and pH 4.5. It is difficult to compare the results of Stratford (1983) with the present work because different pH values were employed in the two studies. However, simple calculations, based on knowledge of proportions of sulphite species present in solution (King *et al.*, 1981; Appendix 1), show that *Sacch. cerevisiae* AWRI 1A65 and *Sacch. cerevisiae* TC8 are inhibited by the presence of 4.6 and 2.3 μM molecular sulphur dioxide, respectively. Although no direct experimental comparison is available, it would appear that pitching yeasts *Sacch. cerevisiae* AWRI 1A65 and *Sacch. cerevisiae* TC8 possess sulphite tolerances of the same order of magnitude and that the former yeast is slightly more resistant to

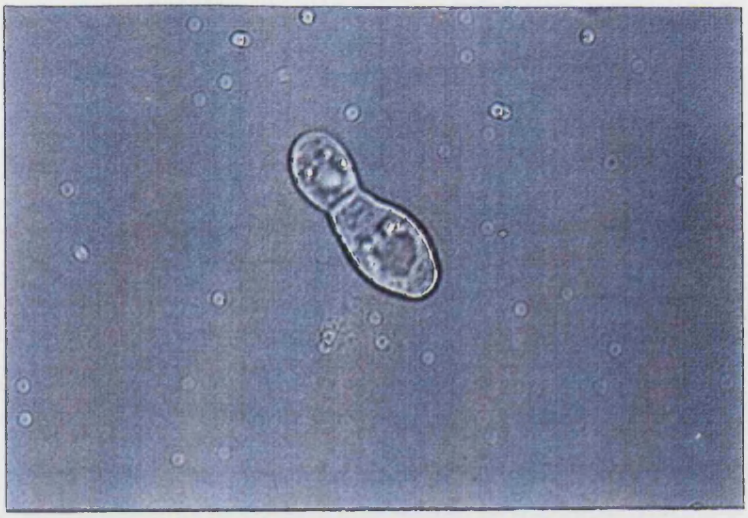
sulphite than the latter. The relative intolerance of *Sacch. cerevisiae* AWRI 1A65 was exhibited during fermentations with concentrate-derived apple juice; growth of the yeast was depressed by free sulphite concentrations greater than approximately 0.3 mM. In view of the many yeasts which are very sensitive to sulphite, for example, strains of *Candida*, *Rhodotorula* and *Pichia* species, it would perhaps be unfair to refer to *Sacch. cerevisiae* AWRI 1A65, and by implication other pitching yeasts, as intolerant organisms. However, there does seem to be a prevailing belief within the cider industry that the pitching yeasts in use are particularly resistant to sulphite. If a scale of sulphite tolerance were constructed to include strains of *S'codes ludwigii* then this belief should be challenged.

Stratford (1983), in his thesis, attributed the relative sulphite tolerance of *Sacch. cerevisiae* TC8, in comparison to *Sacch. cerevisiae* NCYC 366, to the fact that it is used in cider-making, during which sulphite treatment occurs, and some selection for a more tolerant strain would be likely. There might be a temptation to apply this reasoning to *Sacch. cerevisiae* AWRI 1A65. However, selection pressures, as described by Stratford (1983), do not occur. In cider-making, inocula are not recycled from one fermentation to the next, as is the case in ale-industries. Each cider fermentation originates from a slant-culture and, increasingly, organisms used for this purpose have been purchased from large culture collections on the basis of many attributes, and not solely sulphite tolerance. Apart from a speculative comment by Warth (1985), there is no evidence to substantiate the notion that sulphite tolerance can be acquired through exposure to the preservative. Warth (1985) found that some growth curves did not show increases corresponding to depletion of free sulphur dioxide. Unfortunately, he (Warth, 1985) also described growth curves in which corresponding increases did occur, and others in which *decreases* in growth were recorded. The data given by Warth (1985) might be considered unreliable and no substantial proof of yeast adaptation to sulphite can be derived from them.

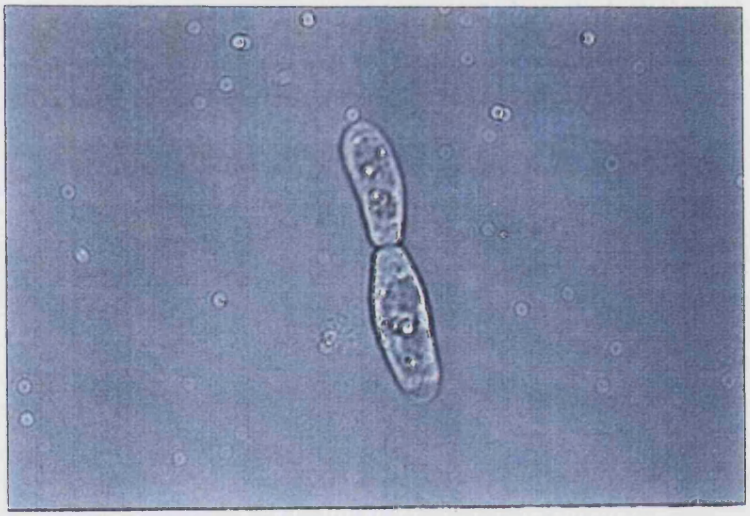
Saccharomyces cerevisiae AWRI 1A65 was used in this study in order to provide a necessary point of reference. It is the yeast which is used to ferment apple juice by HP Bulmer Ltd. in production of all of their ciders and it provided a baseline from which to consider sulphite tolerance. The strains of *S'codes ludwigii* were chosen on an arbitrary basis. *Saccharomycodes ludwigii* BC1 was isolated by workers at the Hereford factory of HP Bulmer Ltd., and preliminary studies indicated it would grow in media containing 500 mg total sulphite l^{-1} (R. Wood, personal communication); it was the most sulphite-resistant strain of *S'codes ludwigii* which had been isolated there. *Saccharomycodes ludwigii* TC10 was studied because it provided a link to previous studies of sulphite tolerance (Stratford *et al.*, 1987). Fortuitously, the two strains of *S'codes ludwigii*, apart from being morphologically dissimilar (Plates 1 - 4), demonstrated slightly different physiological reactions to sulphite.

Saccharomycodes ludwigii is inherently tolerant of sulphite and, because of this, has become legendary throughout the cider industry for its ability to grow in, and spoil, sulphite-containing ciders. Using microtitre-plate analysis to test for sulphite tolerance, a range of reactions was evident with the three yeast strains tested; on this basis alone, *S'codes ludwigii* BC1 was declared most tolerant of sulphite, and *Sacch. cerevisiae* AWRI 1A65 least so. Dissimilarities between the two *S'codes ludwigii* strains, exhibited in microtitre-plate analysis, were less marked in subsequent different experiments involving reactions with sulphite, for example, measurement of sulphite tolerance in one-litre portions of media. The nature of microtitre-plate analysis dictates that cell suspensions used are not so dense that they prohibit A_{600nm} measurement. Cell suspensions of density 0.1 mg dry wt. ml^{-1} were used for microtitre plates, rather than 0.5 mg dry wt. ml^{-1} which were used in one-litre portions of media. The lower cell densities prepared for microtitre analysis might have exaggerated any differences in reaction to sulphite between *S'codes ludwigii* strains so that *S'codes ludwigii* BC1 was perceived to be

1



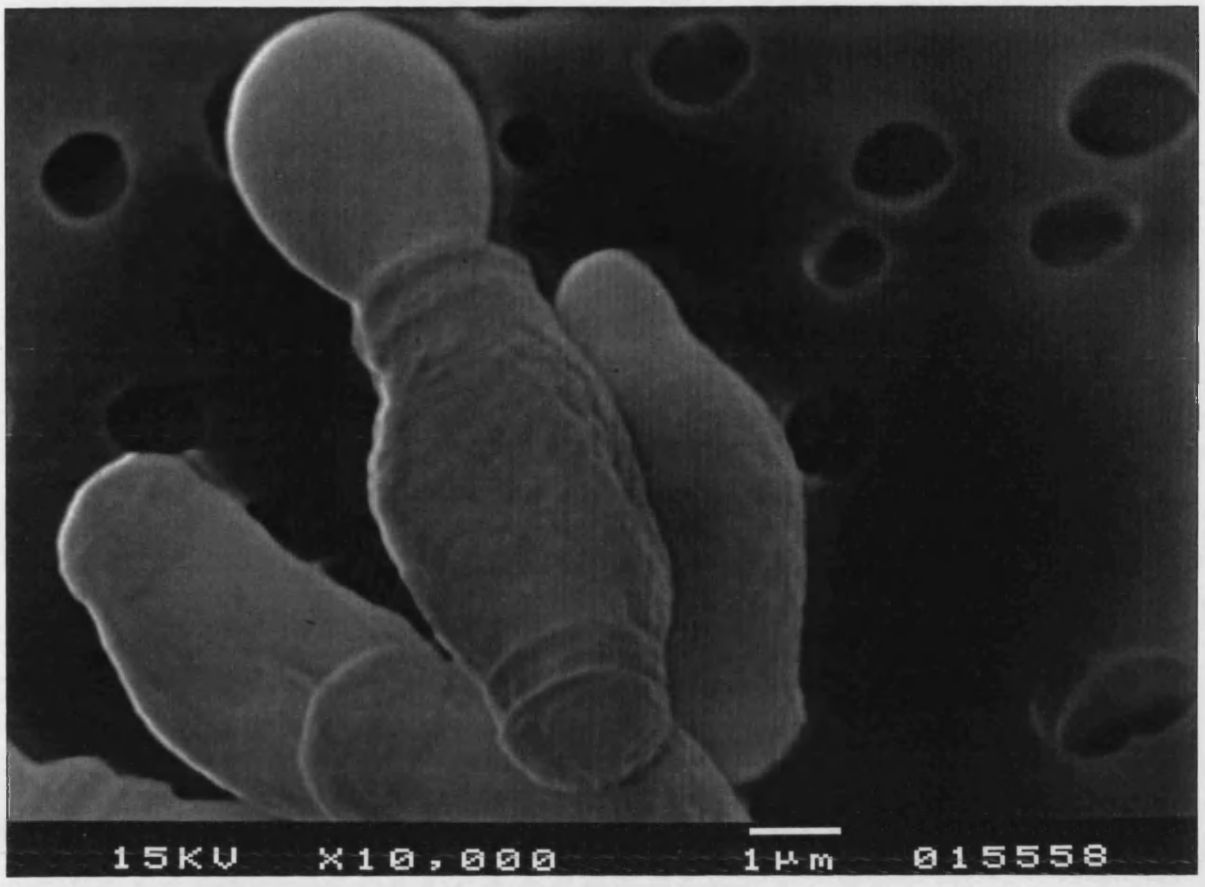
2



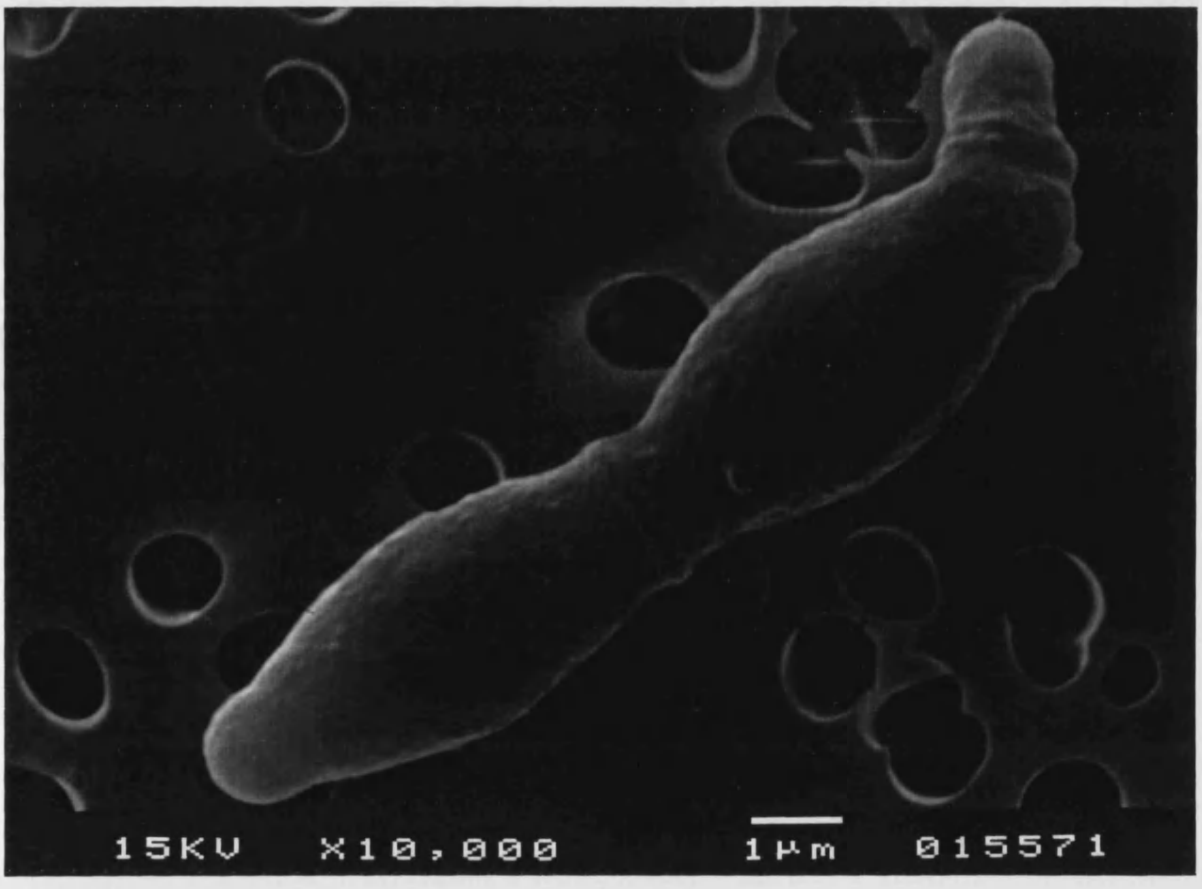
Plates 1 and 2: Typical cells of *Saccharomycodes ludwigii* BC1 (**1**) and *Saccharomycodes ludwigii* TC10 (**2**) as viewed under the light microscope. Mid-exponential cells were photographed using a Zeiss Axioskop 20 Microscope attached to a Brian Reece Optical-Disc Archiving system via a J.V.C. Video Camera and a Mitsubishi Colour Video Copy Processor. Cells were not stained, but pictures were enhanced by using a false light setting available through the Archiving system. Approximate dimensions of cells, displayed above, are 3.5 x 9.0 μm and 3.0 x 13.0 μm for *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10, respectively.

Plates 3 and 4: Scanning electron micrographs of *Saccharomycodes ludwigii* BC1 (**3**) and *Saccharomycodes ludwigii* TC10 (**4**). Organisms were harvested at mid-exponential phase, washed once in distilled water and twice in 1.5 M sorbitol buffer (pH 6.8) containing 20 mM Tris and 10 mM magnesium chloride. Organisms were fixed by suspending in buffer containing 3 % (w/v) gluteraldehyde for 1 h, washed twice in buffer without gluteraldehyde, and resuspended in buffer containing 2 % (w/v) osmium tetroxide for a further hour. Preparations were dehydrated by suspending in 50 % (v/v) ethanol, then 70 % (v/v) ethanol for 10 min each, and finally 3 x 15 min in 100 % ethanol. Preparations were dried in a Polaron E3000 Critical Point Drier, under pressure of carbon dioxide, and attached to conductive stubs coated with liquid graphite. Following coating of preparations with gold, in the presence of argon (Edwards Sputter Coater), structures were photographed via a JEOL 100CX electron microscope.

3



4



substantially more sulphite-tolerant than *S'codes ludwigii* TC10. It would be fair to postulate that this could be explained in terms of yeast-cell morphology. Cells of both yeast strains are essentially bowling-pin shaped (Barnett *et al.*, 1983), but *S'codes ludwigii* TC10 is thinner and more elongated than *S'codes ludwigii* BC1 (Plates 1 - 4). It is conceivable that the larger surface area-to-volume ratio of *S'codes ludwigii* TC10 renders its intracellular sites marginally more susceptible to effects of sulphite, and this was reflected in poorer growth over the six hour period of microtitre-plate analysis.

It was important to define sub-lethal concentrations of sulphite for the three yeast strains. To be able to study physiological effects of sulphite on yeast, it is obviously important not to kill the organisms. Inhibitory effects on yeast cells are reflected in decreased growth; if yeast viability is found to be unaffected despite growth inhibition, then sub-lethal conditions are present. In experiments detailed in this thesis, a known weight of yeast, harvested during the mid-exponential phase of growth, was resuspended in media containing a known concentration of sulphite. Carrying out experiments in this way, rather than by adding sulphite solutions to exponentially growing cultures (Pilkington and Rose, 1988), allows measurement of free sulphite concentrations which are present at the time of yeast addition. Addition of sulphite solutions to exponentially growing cultures results in an instant loss of information; concentrations of free sulphite cannot be determined. Measurements would have to take place at the exact moment of sulphite addition; currently, this is experimentally impossible.

Having defined sub-lethal concentrations, both strains of *S'codes ludwigii* were found to be, in terms of total sulphite concentrations, a little under ten times (9.8 times) more tolerant of sulphite than *Sacch. cerevisiae* AWRI 1A65. In terms of free sulphite, both strains of *S'codes ludwigii* were found to be a little over ten times (11.5 times) more tolerant of sulphite than *Sacch. cerevisiae* AWRI 1A65. This is in general agreement with the relationship between *Sacch. cerevisiae* TC8

and *S'codes ludwigii* TC10 elucidated by Stratford *et al.* (1987). It must be remembered that the boundary between a sub-lethal and a lethal concentration of sulphite is not clearly definable. However, it would be fair to say that a ten-fold difference of tolerance exists between *S'codes ludwigii* strains and *Sacch. cerevisiae* AWRI 1A65.

Acetaldehyde Production by Yeasts.

The experimental regime, already described, allowed accurate correlations of acetaldehyde production with declines in free sulphite concentrations in yeast cultures. Acetaldehyde has been implicated for many years as a major binding component of sulphite (Weeks, 1969), and has been proposed as a means of explaining extreme sulphite tolerance displayed by strains of *S'codes ludwigii* (Stratford *et al.*, 1987). Other compounds which bind sulphite, such as pyruvate and α-ketoglutaric acid, do not appear to be important as micro-organism defence strategies against sulphite (Stratford, 1983). Both *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10 produced enough acetaldehyde to bind all free sulphite which was present upon addition of yeast to media. The data indicate that all decreases in free sulphite concentrations occurred as a direct result of acetaldehyde production by yeasts. Over-production of acetaldehyde by *S'codes ludwigii* TC10, which was reported by Stratford *et al.* (1987), did not occur in these experiments. It would seem most likely that the different methodologies used for acetaldehyde measurement, in this report and that of Stratford *et al.* (1987), account for this inconsistency. The multiple-step steam-distillation method for determining acetaldehyde concentrations, detailed by Stratford *et al.* (1987), would arguably have been more prone to experimental error than the method used in the present study. However, no experiments were carried out to directly compare the results of Stratford *et al.* (1987) with those obtained through use of Boehringer Mannheim kits in this study.

Over-production of acetaldehyde by yeasts cannot be explained in terms of Neuberg's third form of fermentation; acetaldehyde should be produced in a concentration equimolar to the concentration of free sulphite present. Excessive production of acetaldehyde might imply a stimulation of metabolic activity by sulphite. However, all evidence is to the contrary, as it is well-known that yeast metabolism is impaired by sulphite (Hinze and Holzer, 1985; Maier *et al.*, 1986). Alternatively, over-production may imply that acetaldehyde-bisulphite complexes dissociate, thereby releasing free sulphite which further binds to acetaldehyde. Stratford and Rose (1985) demonstrated that acetaldehyde-bisulphite complexes do indeed dissociate, and that the separate components enter yeast cells at different rates. However, acetaldehyde-bisulphite complexes are relatively very stable, and dissociation of complexes would most likely occur through metabolism of one or both components. Also, dissociation of complexes would not necessarily increase acetaldehyde concentrations, since overall pool-size of acetaldehyde would not change. The current data do away with the awkwardness of having to explain over-production of acetaldehyde.

Saccharomyces cerevisiae AWRI 1A65 appeared to produce less acetaldehyde than was required to bind all free sulphite initially present. The concentrations of acetaldehyde and sulphite measured in experiments with *Sacch. cerevisiae* AWRI 1A65 were ten times lower than those quantified in experiments with *S'codes ludwigii* strains. Accuracy of measurement of acetaldehyde may have been decreased at such low concentrations; this possibility was reflected by the relatively large standard deviations obtained in experiments with *Sacch. cerevisiae* AWRI 1A65. However, it is possible that a very real effect was observed, in which case *Sacch. cerevisiae* AWRI 1A65 under-produced acetaldehyde. Sulphite so affects metabolism of *Sacch. cerevisiae* AWRI 1A65 that acetaldehyde production is partially inhibited. Indeed, of the three strains, *Sacch. cerevisiae* AWRI 1A65 showed greatest inhibition of ethanol production. Such inhibition of acetaldehyde, and therefore ethanol production, would arise 'upstream' in metabolism, most likely

through inhibition of glyceraldehyde-3-phosphate dehydrogenase, which has been identified by Hinze and Holzer (1985) as being particularly sensitive to sulphite. This implies that glyceraldehyde-3-phosphate dehydrogenase of *Sacch. cerevisiae* AWRI 1A65 is more sensitive to sulphite than the corresponding enzyme of *S'codes ludwigii* strains. This must remain speculative; a more refined assay for acetaldehyde would be required to establish exact sulphite-acetaldehyde relationships for *Sacch. cerevisiae* AWRI 1A65.

Ethanol production by yeasts, in the presence of sulphite, has already been touched upon. With all three yeast strains, ethanol production was decreased, though not halted, by sub-lethal concentrations of sulphite. *Saccharomyces cerevisiae* AWRI 1A65 showed the greatest decrease in ethanol production, and *S'codes ludwigii* BC1 the least. This corresponds with the general indications that *S'codes ludwigii* BC1 is slightly more sulphite-tolerant than *S'codes ludwigii* TC10, and that both *S'codes ludwigii* strains are more tolerant than *Sacch. cerevisiae* AWRI 1A65. A decrease in ethanol production in favour of acetaldehyde production has previously been demonstrated with *Sacch. cerevisiae* TC8, *Zygosacch. bailii* NCYC 563 and *Zygosacch. bailii* NCYC 1427 (Pilkington, 1988).

An important point to make regarding acetaldehyde production by yeasts, and in particular by strains of *S'codes ludwigii*, is that the phenomenon should not be considered to be a yeast defence mechanism in its own right. A distinction between producing acetaldehyde in order to survive, and being able to produce acetaldehyde as a result of survival, must be made. A yeast cell is only able to produce acetaldehyde to bind sulphite if metabolism is not halted by inhibitory effects of the preservative. Acetaldehyde production by yeasts is not a positive defence mechanism, it is an incidental one. With this in mind, the most important investigation to be carried out is to find the means by which strains of *S'codes*

ludwigii are able to survive and metabolize, and thereby produce acetaldehyde, in concentrations of sulphite which kill *Sacch. cerevisiae* AWRI 1A65.

Sulphite Accumulation by Yeasts.

There is little doubt that sulphite enters cells of *Sacch. cerevisiae* and *S'codes ludwigii* by free diffusion (Stratford and Rose, 1986; Stratford *et al.*, 1987). Stratford *et al.* (1987) also demonstrated that *Sacch. cerevisiae* accumulates substantially more sulphite than does *S'codes ludwigii*; data presented in this thesis confirm this. It is an irrevocable fact that the considerable sulphite resistance shown by strains of *S'codes ludwigii* arises because of their relatively small intracellular accumulation of the preservative. This thesis has provided, for the first time, maximum sulphite-accumulation values. *Saccharomycodes ludwigii* BC1 accumulated slightly more sulphite than did *S'codes ludwigii* TC10; 27.7 and 19.3 nmol (μl intracellular fluid)$^{-1}$, respectively. *Saccharomyces cerevisiae* AWRI 1A65 accumulated a maximum concentration of sulphite [82.8 nmol (μl intracellular fluid)$^{-1}$] which was around three times that accumulated by *S'codes ludwigii* BC1. In contrast, Stratford (1983) found that, at concentrations of sulphite which were lethal to cells, intracellular sulphite concentrations were very similar in *Sacch. cerevisiae* TC8 and *S'codes ludwigii* TC10. However, Stratford (1983) referred only to 'lethal' concentrations, and did not declare maximum accumulation values. Therefore, a comparison between values given by Stratford (1983) and those presented in this thesis would be meaningless because of the ambiguity associated with interchange of the terms 'lethal' and 'maximum'.

Both strains of *S'codes ludwigii* accumulated sulphite over two distinct phases, up to concentrations which represented maximum accumulation values. Rates of accumulation were greatest during the second phase, and were associated with a decrease in viability of yeast suspensions. Onset of the second phase required a higher concentration of sulphite with *S'codes ludwigii* BC1 than it did with *S'codes ludwigii* TC10. Only a single phase of accumulation was observed

with *Sacch. cerevisiae* AWRI 1A65. It is apparent that the nature of sulphite accumulation is markedly different between the species *Sacch. cerevisiae* and *S'codes ludwigii*. Uptake of sulphite over two phases has not previously been reported for *S'codes ludwigii*. Warth (1989) found that high concentrations of propionic acid increased the rate of accumulation of this weak acid by *Zygosacch. bailii*. He (Warth, 1989) attributed this effect to membrane disruption caused by high concentrations of propionic acid. It is possible that two-phase accumulation of sulphite by *S'codes ludwigii* is analogous to this. This would mean that, up to a threshold concentration of sulphite, yeast cell membranes would act as barriers to diffusion of sulphur dioxide; disruption of barriers would be evidenced by onset of the second phase of uptake. Warth (1989) did not indicate whether higher rates of propionic acid accumulation by *Zygosacch. bailii* were reached gradually, or after a well-defined switch as was observed with *S'codes ludwigii* in the present work. However, disruption of cell membranes not only implies an increased accumulation of sulphite, but also that leakage from cells would occur. Higher rates of sulphite accumulation would not necessarily be observed if leakage were in evidence, though this would depend on influx-efflux ratios of sulphite. In the present study, it would seem that membrane disruption, if it occurred during the second phase of uptake, resulted in a greater influx of sulphite than efflux. Stratford (1983) reported sulphite-induced inhibition of amino-acid transport, but thought it an unlikely explanation that plasma-membrane integrity was altered so that solutes would leak out. Certainly, membrane disruption does become apparent after maximum values of sulphite accumulation are attained, as evidenced by decreases in intracellular sulphite concentrations; this could only have been caused by sulphite leakage from cells.

To explain the two-phase accumulation phenomenon observed with *S'codes ludwigii* strains, a system involving active anion export might, on the surface, appear to be an attractive solution. Up to the onset of the second phase, at which point the system collapses, an active transport system might operate to export anions

from the cell. However, for the reason that such systems would require continuous and unreasonable expenditures of energy, it has now been generally accepted that they do not function in preservative-yeast interactions (Cole and Keenan, 1987; Pilkington, 1988). As yet, there is no water-tight explanation for two-phase accumulation of sulphite by *S'codes ludwigii*, but a hypothesis to fit all known facts will be presented later in this Discussion.

Intracellular pH Values of Yeasts.

Is it possible to measure yeast intracellular pH values? The notion of an intracellular pH value is an absurd one. Within a single yeast cell there will be a myriad of pH values, since the pH optima for different segments of metabolism will not be the same. Nevertheless, efforts are continually made to find a definitive method for intracellular pH value measurement. Distribution of weak acids, including propionic acid, between intracellular and extracellular environments of yeast suspensions are the most common methods employed to measure intracellular pH values (Conway and Downey, 1950; Krebs *et al.*, 1983; Cole and Keenan, 1987; Pilkington and Rose, 1988). Freeze-thaw methods have also been commonly used (Conway and Downey, 1950; Warth, 1988). This procedure involves freezing portions of yeast in liquid nitrogen, thawing, and re-freezing in the same manner. The process is repeated about six times, and a flat glass electrode is used to measure pH values of the liquid released from freeze-thawed yeast. This method could not be used to measure intracellular pH values of organisms suspended in buffer; therefore, it could not be used in conjunction with sulphite-accumulation experiments where control of extracellular pH value was critical. A technique used by Cole and Keenan (1987) in which cells were treated with fluorescein diacetate, in order to relate measured fluorescence to intracellular pH values, was dismissed as completely unreliable by Pilkington (1988). As will be discussed later, measurements of intracellular pH values are very much dependent on the methods used to obtain them. With this in mind, there was no hesitation in choosing

propionic acid distribution to measure intracellular pH values in this work, in order that results could be related to those acquired commonly by other workers.

Stratford *et al.* (1987) hypothesized that, on the basis of its greater sulphite resistance, *S'codes ludwigii* would have a lower intracellular pH value than *Sacch. cerevisiae*. Data presented in this thesis are the first to prove that this hypothesis is correct. A relatively high intracellular pH value is associated with sulphite sensitivity, while a relatively low intracellular pH value is associated with sulphite tolerance. Stratford (1983) proposed that a difference in intracellular pH value of one unit between *Sacch. cerevisiae* and *S'codes ludwigii* would be required in order to explain their different sulphite tolerances solely on the basis of intracellular pH values. Harvested in the mid-exponential phase of growth, intracellular pH values of both *S'codes ludwigii* strains were found to be 0.27 units lower than those of *Sacch. cerevisiae* AWRI 1A65, as calculated from propionic acid-uptake data. Assuming equal and constant buffering capacities, for a given extracellular sulphite concentration and pH value, it can be calculated that *Sacch. cerevisiae* AWRI 1A65 would, theoretically, accumulate approximately one and a half times more sulphite than would either strain of *S'codes ludwigii*. In practice, however, it was found that *Sacch. cerevisiae* AWRI 1A65 accumulated approximately ten times more sulphite than did either *S'codes ludwigii* strain. From this information, it would appear that sulphite accumulation is not merely a function of average intracellular pH value, and some other factor must exert an important influence; intracellular buffering capacities, which heavily influence sulphite uptake by yeast cells, are explored in a following section.

Stationary-phase cells of all three strains possessed the lowest intracellular pH values of growth phases studied. This is interesting from the point of view that stationary-phase organisms are thought to be the most resilient of cells from different phases of growth. In keeping with this, stationary-phase cells generally accumulated least sulphite. These findings are in agreement with the work of

Tracey Holland (unpublished data) who investigated benzoate tolerance in *Zygosacch. bailii*, and found that benzoate accumulation was lowest during the stationary phase of growth; this, in turn, was correlated with relatively low intracellular pH values. Following on from this, two of the three strains studied in the present work, namely *Sacch. cerevisiae* AWRI 1A65 and *S'codes ludwigii* BC1, would be most vulnerable to the effects of sulphite during early-exponential phase on the basis of relatively high intracellular pH value measurements. Certainly, maximum lethal effects from a given concentration of sulphite would be gained, with all three yeast strains, by treating early- or mid-exponential phase organisms rather than stationary-phase organisms. Although cider-making constitutes a much more complicated system than that described in the present study, these observations should be taken into consideration. Ensuring a transition from stationary to early-exponential phases of growth, of wild yeasts present in apple juice, would make sulphite-supplementations more efficacious.

There is one value of intracellular pH for *S'codes ludwigii* in the literature (Warth, 1988); measurements of intracellular pH value made in the present work are not in agreement with this, nor with values given for *Sacch. cerevisiae* in the same report. Warth (1988) obtained intracellular pH values, using a freeze-thaw method, of 7.2, 6.9 and 7.1, respectively, for *Sacch. cerevisiae* FRR 1297, *Sacch. cerevisiae* FRR 1298 and *S'codes ludwigii* 1555. Unfortunately, he (Warth, 1988) did not indicate the growth phase at which these organisms were harvested; as this thesis demonstrates, cells from different growth phases can have intracellular pH value differences of the order of 0.2 - 0.3 units. Despite this shortcoming, the intracellular pH value for *S'codes ludwigii* 1555, in particular, does not make sense in light of the findings here. Assuming that *S'codes ludwigii* 1555 was harvested at mid-exponential phase, the published intracellular pH value is 0.36 units greater than those given in this thesis for *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10. To compound the lack of information regarding growth phase, Warth (1988) made

no reference to buffering capacity and, as will be described later, intracellular pH values are considerably more valuable if intracellular buffering-capacity information is also available.

Interpretation of Intracellular pH Values.

If the concentration of sulphite accumulated by yeasts is determined by intracellular pH values, then it follows that sulphite accumulation data can be used to estimate intracellular pH values. Weak acids of one kind or another are the most common form of reagent used to measure intracellular pH values in yeast, as was described previously in this Discussion. Calculations detailed in this thesis are the first to use sulphite as a means of producing values for intracellular pH in the presence of sulphite. Intracellular pH values calculated from sulphite-uptake data were found, with all three yeast strains, to be considerably lower than those calculated from propionic acid-uptake data. For example, sulphite accumulation values of 5.1, 4.7 and 4.7 nmol (mg dry wt.)$^{-1}$ for *Sacch. cerevisiae* AWRI 1A65, *S'codes ludwigii* BC1 and *S'codes ludwigii* TC10, respectively, gave intracellular pH values, calculated from the same sulphite uptake-data, of 5.94, 5.03 and 4.91, respectively. For the same experiments, intracellular pH values calculated from propionic acid-uptake data were 6.95 for *Sacch. cerevisiae* AWRI 1A65, and 6.74 for both *S'codes ludwigii* strains. In this example, intracellular pH values calculated from sulphite-uptake data are 1.01 - 1.83 units lower than those calculated from propionic acid-uptake data. The methods used for quantifying intracellular pH values appear to be measuring entirely different entities. Warth (1977) made a similar observation; exact correspondence was not found between intracellular pH values as measured using a freeze-thaw method, and calculated from weak acid-accumulation data; a greater accumulation of weak acid was expected but not obtained. Results given in this thesis, and the observations of Warth (1977), indicate that yeast cells either accumulate much less sulphite than

would be calculated from intracellular pH values obtained by conventional means, or that methods used to measure intracellular pH values produce over-estimates.

At this point, it must be stated that the method used to calculate intracellular pH values from sulphite-uptake data has faults, as a number of important factors were neglected or simplified. Binding of sulphite, both intracellularly and extracellularly, was ignored. Without doubt, extracellular free sulphite concentrations, which were predicted as part of intracellular pH value calculations, would have been over-estimates; binding would have accounted for a considerable proportion of extracellular sulphite. Binding of sulphite would also have been likely at the cell wall and plasma membrane, and most certainly within the yeast cell. However, intracellular binding of sulphite would not explain why pH values were obtained which were lower than expected. A considerable amount of sulphite-binding within a cell would serve only to draw further amounts of sulphite from the extracellular environment. A sink effect would be created, the magnitude of which would determine values of intracellular pH obtained; the greater the accumulation of sulphite, the higher would be the measurement of intracellular pH value.

Compartmentalization within cells should not be overlooked as an explanation for disparate intracellular pH-value measurements by different methods; fates of propionic acid and sulphite entering a yeast cell will not be the same. Furthermore, there is potential for permeability of the plasma membrane to preservative anions; Warth (1977) attributed his disparate results, described already, to this. Combining the case for plasma-membrane permeability to anions with that of compartmentalization, it could be argued that, because the fate of propionic acid and sulphite within a cell are likely to be different, sulphite anions or molecular sulphur dioxide may be more freely able to cross the plasma membrane than are propionate anions or propionic acid. Use of sulphite to measure intracellular pH values may result only in measurements of the outer region of the cell, and not an average value for the whole cell; this would explain the relatively low intracellular pH values obtained from sulphite-uptake data. Conway and Downey (1950)

attempted to measure pH values of the outer regions of cells, effectively yeast cell walls, using glyceric acid solutions; they found that the pH values there were the same, or nearly the same, as those for the whole cell. Equilibrium of molecular sulphur dioxide across the plasma membrane would therefore depend on a similar equilibrium across the cell wall. The results discussed here indicate that equilibrium between intracellular and extracellular sulphite may occur as a localized event, and that sulphite does not necessarily penetrate the whole yeast cell.

As a conclusion to this section, it should be recognized that values for intracellular pH are functions of the methods used to obtain them, and there is no definitive way of measuring such phenomena. Until a standard method of measuring yeast intracellular pH values becomes accepted, it will be very difficult to interpret data obtained with different techniques by different workers.

Buffering Capacities of Yeasts.

Buffering capacity measurements were initiated by studying the effects of acid on whole cells, then a study of fractions of whole cells was carried out and, finally, intracellular buffering capacity was investigated; an extracellular to intracellular approach was adopted.

A very curious effect was observed when portions of hydrochloric acid were added to unbuffered yeast suspensions. It was found that suspension pH values were buffered by the presence of *S'codes ludwigii* strains, but not by *Sacch. cerevisiae* AWRI 1A65. Buffering effects were not observed when non-viable *S'codes ludwigii* were employed in similar experiments. It appears as if viable *S'codes ludwigii* are able to influence their environment such that, in the presence of acid, a decline in pH value is resisted; viable *Sacch. cerevisiae* AWRI 1A65 are not capable of comparable resistance. Either *S'codes ludwigii* is responsible for removing protons from suspension, or for production of compounds which serve to neutralize acid. It is unlikely that *S'codes ludwigii* absorbs protons; a yeast cell continually expends energy exporting protons from the cell in order to maintain a

proton-motive force. Export of hydroxyl anions, to neutralize extracellular acid, would have to be compensated for by exchange with some other anion; a putative chloride cycle is described by Booth (1985). However, the nature of the buffering exhibited by *S'codes ludwigii* is as yet unknown. It does not originate within the composition of the cell wall, nor in any components external to the cell wall, as data presented in this thesis demonstrates. The cell wall does possess considerable buffering capacity (Cartwright *et al.*, 1986), but a buffering capacity which was found, in the present work, to be equal in all three yeast strains tested. Purely from the point of view of resistance to sulphite, yeast cells would accumulate smaller concentrations of sulphite if extracellular pH values were close to intracellular values; intracellular-extracellular pH value differentials, which determine the degree of sulphite accumulation, would be minimized. However, this is a very simplistic view since acidification of yeast suspensions occurs immediately a carbon source becomes available. Also, yeast optimum growth occurs in an acidic pH range, roughly 4.0 - 6.0. Furthermore, all experiments which demonstrated superior sulphite tolerance of strains of *S'codes ludwigii* over *Sacch. cerevisiae* AWRI 1A65, apart from those investigating intracellular buffering capacity, were carried out at pH 4.0, and this would have negated any potentially advantageous buffering effects exerted by yeasts. It can be imagined that, in certain environments of low nutrient status and sulphite-presence, a capacity to raise the pH value of its environment would enhance survival of *S'codes ludwigii*. The significance of this property, with regard to cider-spoilage, is likely to be minimal; in terms of surviving sub-optimal conditions and persisting as potential sources of infection, such a buffering effect might be more important.

Pilkington and Rose (1988) indicated that intracellular buffering capacities of different yeasts were not the same, but did not speculate as to the significance of this. It must be admitted here that the hypothesis originally developed, for the present work, to link intracellular buffering capacity and sulphite tolerance was

incorrect. Preliminary (unpublished) data obtained by Tracey Holland, in experiments with benzoate tolerance in *Zygosacch. bailii* and *Sacch. cerevisiae*, indicated that the superior tolerance of the former yeast might be related to an ability to control intracellular pH values; *Zygosacch. bailii* showed much less fluctuation in intracellular pH value than did *Sacch. cerevisiae*. Cole and Keenan (1987) indicated that preservative resistance of yeasts might be attributable to greater intracellular buffering capacities. Thus, prior to experimentation, it was decided that the yeast best able to buffer its internal environment, and so maintain optimum pH values for metabolism, would be best able to tolerate sulphite. Titration of cell cytosols dispelled this notion; *S'codes ludwigii* strains possessed smaller intracellular buffering capacities than did *Sacch. cerevisiae* AWRI 1A65. Subsequently, a new hypothesis was developed which was more logical in terms of minimizing sulphite accumulation in yeast cells, but illogical if constancy of intracellular environments are considered to be critical to yeast-cell survival. A relatively small intracellular buffering capacity would provide little resistance to acidification by sulphite anions, and intracellular pH values would decline. Such declines in intracellular pH values would increase the relative proportion of molecular sulphur dioxide present within yeast cells; intracellular-extracellular pH value differentials would become smaller so that sulphite accumulation would be minimized. Consequently, sulphite resistance derived in this fashion would necessitate a capacity for yeast cells to survive despite relatively low intracellular pH values. On the other hand, maintenance of relatively high intracellular pH values through relatively large intracellular buffering capacities would serve only to concentrate sulphite within the cell. Resistance to decline in intracellular pH value would maintain relatively low concentrations of molecular sulphur dioxide so that further preservative would be drawn into yeast cells. This hypothesis was found to be correct in experiments using radioactively-labelled sulphite or propionic acid, and these are discussed in a following section.

A brief discussion of the method used to isolate cell cytosols, for experiments which were briefly mentioned above, is given here. Yeast cells were disrupted with ultrasound (sonication), rather than by homogenization with glass beads which has now become a standard technique for yeast breakage (Campbell and Duffus, 1988). Sonication incurred fewer dilution steps than homogenization; it was decided that it was not acceptable to dilute a suspension more than absolutely necessary in a system where buffering-capacity changes due to dilution were not predictable. Mixtures of glass beads and disrupted cells are unavoidably diluted when removing them from Braun bottles. DNA was chosen as the marker for intact yeast cells, as a molecule not normally found in extracellular environments.

Results from experiments measuring uptake of [^{35}S]sulphite and [2-^{14}C]propionate were presented in this thesis in such a way as to provide indications of intracellular buffering capacities; such information has not been presented in this way before, but its value is immediately evident. *Saccharomyces cerevisiae* AWRI 1A65 was found to possess a larger intracellular buffering capacity than either *S'codes ludwigii* BC1 or *S'codes ludwigii* TC10; this was in general agreement with direct titration of cell cytosols against acid, which has already been described. On acidification of cytoplasm by sulphite, intracellular pH values of *S'codes ludwigii* are lowered to a considerably greater extent than those of *Sacch. cerevisiae* AWRI 1A65. For example, after accumulation by *Sacch. cerevisiae* AWRI 1A65 of sulphite concentrations approximately twice those accumulated by *S'codes ludwigii* strains, intracellular pH values of the former yeast remain more than one pH unit higher than those of the latter. A difference in intracellular pH value of one unit between *Sacch. cerevisiae* AWRI 1A65 and *S'codes ludwigii* strains amounts to a ten-fold difference in intracellular-extracellular sulphite differentials, *i.e.* for the same extracellular sulphite concentration, the former yeast would have to accumulate ten times more sulphite than the latter to obtain the same intracellular concentration of molecular sulphur dioxide. In terms of sulphite tolerance, this is

not coincidental. Cole and Keenan (1987) commented that resistance of *Zygosacch. bailii* to weak-acid preservatives was partly due to its ability to tolerate large intracellular pH value decreases. This is certainly true of *S'codes ludwigii*; for the organism to benefit from a small intracellular buffering capacity, an ability to remain viable despite low intracellular pH values would, unquestionably, be essential.

Intracellular pH values, taken in isolation, are not a good means of declaring whether or not an organism is sulphite-tolerant. Intracellular pH values should be considered as fluid, and not fixed, measurements. When considering sulphite tolerance, the capacity for intracellular pH value change is equally important, possibly more important, than single pH value measurements recorded without due consideration of buffering capacity. A poor capacity to buffer its cytoplasm, coupled with an ability to survive large decreases in intracellular pH value, would make an organism an excellent candidate to display sulphite tolerance.

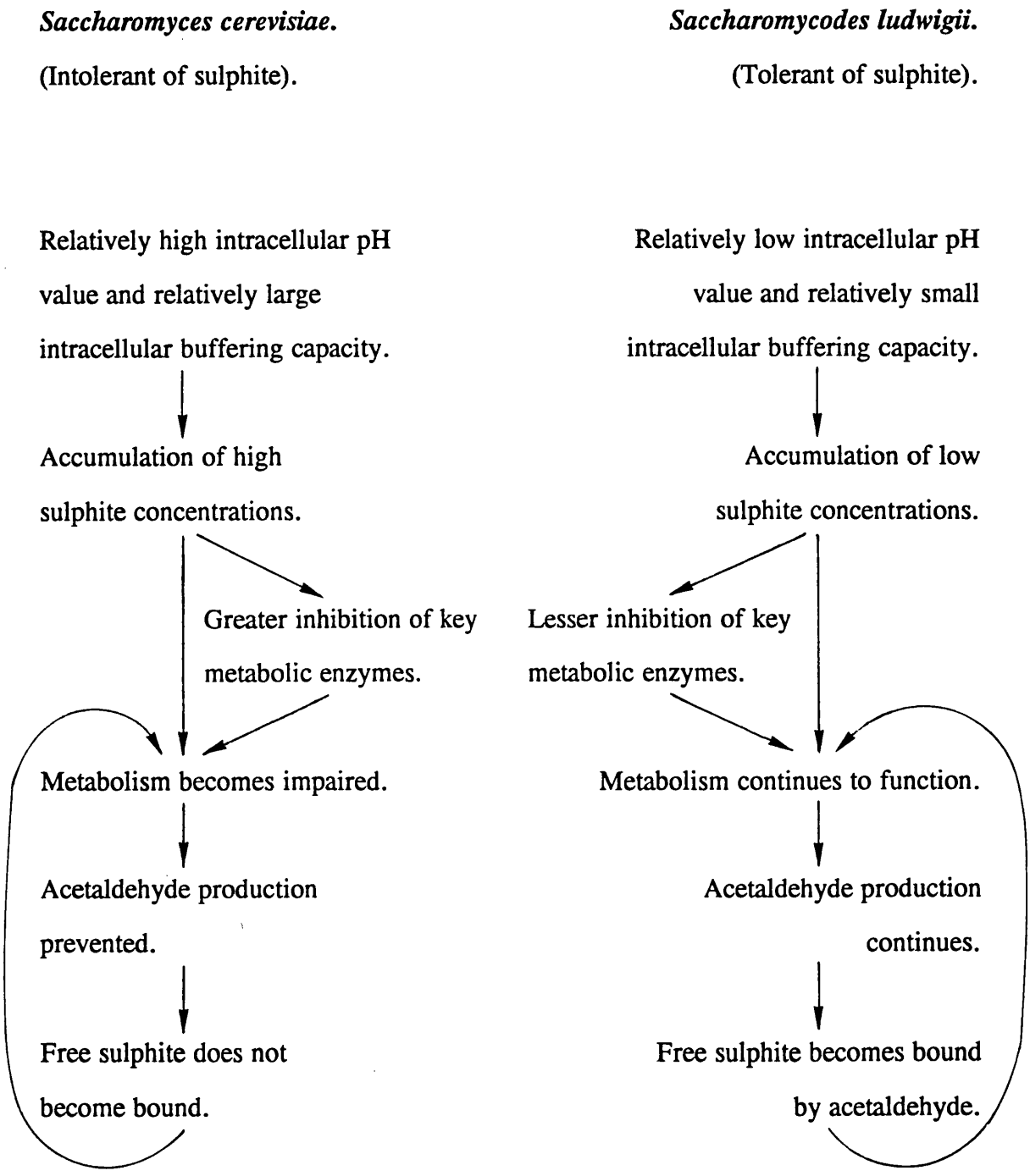
Intracellular buffering capacities for all three strains were found to be greatest during the early-exponential phase of growth. With the exception of *S'codes ludwigii* TC10, smallest intracellular buffering capacities were found during the stationary phase of growth. In addition to the correlation between lower initial intracellular pH values of organisms harvested from different phases of growth with decreased accumulation of sulphite, evidence suggests that intracellular buffering capacities exert a further effect. Generally, it would appear that resting cells from stationary-phase cultures have lower intracellular pH values and smaller intracellular buffering capacities. These two factors are of critical importance when considering tolerance of sulphite. In any phase of growth, strains of *S'codes ludwigii* were found to have smaller intracellular buffering capacities than *Sacch. cerevisiae* AWRI 1A65; at no point in the growth cycle does the latter yeast approach the superior sulphite tolerance of the former yeasts. This ties in with the accepted

notion of stationary-phase cells being the most resilient of a micro-organisms growth cycle.

The informative graph produced from propionic acid-uptake measurements of intracellular pH value combined with sulphite uptake-data is confounded by the corresponding graph consisting of sulphite-uptake measurements of intracellular pH value combined with sulphite-uptake data. Intracellular pH values of the latter graph remain essentially constant within a range of approximately 0.5 pH units. Such an effect is attributable to compartmentalization of sulphite; the preservative does not dissociate evenly within yeast cells, and this has already been discussed. Although a substantial decline in intracellular pH value is measurable using propionic acid distribution, the same is not true when sulphite is used for the same purpose; intracellular distribution of the two acids in question is quite different. The implications of these effects are very important in the grand scheme of preservative tolerance; this becomes evident in the following section.

Hypothesis to Fit All Known Facts.

Sulphite enters cells of *Sacch. cerevisiae* AWRI 1A65 and *S'codes ludwigii* by simple diffusion. Acidification of cytoplasm occurs, but is localized to the plasma membrane region. Such acidification is most apparent in cells of *S'codes ludwigii*, due to their relatively small intracellular buffering capacities. Localized acidification at the plasma membrane allows an equilibrium of molecular sulphur dioxide to be formed across the plasma membrane without creating a sink effect. Thus, cells would be found to accumulate less sulphite than their average intracellular pH value would suggest. The nature of greater sulphite accumulation by *Sacch. cerevisiae* AWRI 1A65 lies in its relatively high intracellular buffering capacity which resists decreases in intracellular pH values and creates the aforementioned sink effect. Due to relatively small intracellular buffering capacities, strains of *S'codes ludwigii* accumulate relatively small concentrations of



Flowchart summarizing the different reactions of *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii* to presence of intracellular sulphite.

sulphite. Consequently, metabolism is able to function, and sulphite-binding compounds, most notably acetaldehyde, can be produced. On the other hand, *Sacch. cerevisiae* AWRI 1A65 accumulates sufficient sulphite to seriously impair or shut down metabolism; acetaldehyde is not produced, sulphite does not become bound, and lethal effects of the preservative become apparent. In cells of *S'codes ludwigii*, at some concentration of sulphite, localized acidification is overwhelmed and an increased rate of sulphite uptake is observed; the second phase of accumulation, as described earlier, becomes evident. Accumulation of sulphite by *Sacch. cerevisiae* AWRI 1A65, for reasons given above, is sufficiently impressive to make a two-phase uptake phenomenon, if this occurs at all, too subtle to be detectable. In both *Sacch. cerevisiae* AWRI 1A65 and strains of *S'codes ludwigii*, intracellular sulphite concentrations eventually become too great for cells to function. Plasma-membrane disruption occurs and is evidenced by leakage of anions from cells; decreases in intracellular sulphite are measured. A flow-chart summarizing the dissimilar reactions to sulphite of *Sacch. cerevisiae* and *S'codes ludwigii* is presented on the opposite page.

Sulphite Tolerance in the Wider World.

Everything described in this Discussion so far, though it may seem far-removed, was inspired by the cider industry. *Saccharomycodes ludwigii* poses a considerable problem for cider-manufacturers. Maximum permitted concentrations of sulphite, currently 200 mg l^{-1} (3.12 mM), will not suppress growth of this organism. Ongoing discussions within the European Economic Community are generally aimed at decreasing the amounts of preservatives in human foodstuffs; sulphite-containing alcoholic beverages form part of these discussions. Pressure is being brought to bear on cider-manufacturers to reduce concentrations of sulphite currently in use. What would be the consequences of lowering maximum permitted concentrations of sulphite in cider? Results of experiments, presented in this thesis, show that the cider industry could, if concentrate-derived apple juice were used in

preference to fresh juice, safely reduce total concentrations of sulphite in general use to 50 mg l^{-1} (0.78 mM). The clarification process prior to concentrating is known to lower microbial counts in apple juices to approximately 3% of the original juice (Beech, 1993). Artificially increasing microbial loads of concentrate-derived apple juices resulted in a requirement for higher sulphite concentrations in order to produce organoleptically acceptable ciders. Noticeably, no fermentation carried out in these studies was axenic, and pitching yeast-domination of fermentations was not necessarily a pre-requisite for production of palatable ciders. As the cider-industry strives ever-closer to the axenic conditions now *de rigueur* in ale and lager industries, contributions of non-pitching yeasts, in terms of odour and flavour of finished products, should first be assessed.

Fermentations carried out with fresh apple juice were faster than corresponding fermentations with diluted apple-juice concentrate. Fresh juice fermentations were relatively independent of sulphite addition, in contrast with diluted-concentrate fermentations which were influenced to a considerable extent by initial sulphite additions. Fresh apple juice does not necessarily provide a more nutritious medium, for enhancement of pitching-yeast growth, than concentrate-derived apple juice; diluted concentrates are regarded to be as fermentable as the juices from which they were made (Beech and Carr, 1977). It is possible that, because of the relatively high microbial load of fresh apple juice, fermentations had already initiated prior to inoculation with the pitching yeast; this would explain the relatively rapid completion of such fermentations.

Saccharomycodes ludwigii was not isolated from apple-juice fermentations, or from microbiological surveys of the HP Bulmer factory, and this was initially most surprising. Media used for isolation procedures were W.L.N. and Lysine agars and, on occasion, M.Y.G.P. agar. Two of the three media types, namely W.L.N. and M.Y.G.P. agars, would have been expected to support growth of even

the most stressed of *S'codes ludwigii* cells. Each isolate, once resuscitated, was tested on media containing sulphite, which is probably the best means of presumptively identifying *S'codes ludwigii*, and also examined using light microscopy. It is unlikely that inadequate methods of isolation were responsible for failure to detect this organism.

Reports in the literature indicate that *S'codes ludwigii*, in comparison to other yeasts such as *Rhodotorula*, *Hansenula* and *Candida* species, has rarely been isolated from natural environments. Beech (1993) reported that *S'codes ludwigii* has been found on mature cider apples, and in insect frass found in oak trees. It is, however, an organism which is generally regarded to be indigenous to cider factories (R. Wood, personal communication). Perhaps the prevalence of this organism has been overestimated by those in the cider industry. Infections in cider due to *S'codes ludwigii* can be disastrous; sulphite used to prevent spoilage does not stop growth of this yeast, such that even low numbers of organisms can spoil the beverage. It is probable that, in light of the difficulty of isolation, *S'codes ludwigii*, like *Sacch. cerevisiae*, is particularly rare in nature (Vaughan Martini and Martini, 1989). This being the case, good factory hygiene should always prevent *S'codes ludwigii* from arising and causing serious problems in apple juices and ciders.

Darwin would have appreciated the opportunism of *S'codes ludwigii* as an excellent example of evolution at work. A man-made environment of acid environments, containing preservatives such as sulphite, provide ideal conditions for this organism to dominate others. Without such environments, which were created for wine and cider-making, *S'codes ludwigii* might have gone unnoticed. A relatively poor cytoplasmic buffering capacity, a relatively low intracellular pH value, an ability to continue metabolizing in the presence of sulphite in order to produce acetaldehyde and, no doubt, other factors yet to be discovered, allow this organism to flourish.

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APPENDICES.

```
10 REM * SP Maloney *
20 REM * Sulphite Proportions *
30 MODE 0: @%=&00309
40 INPUT "YEAST I.D.    ";N$
50 PRINT: PRINT
60 INPUT "HOW MANY VALUES    ";V
70 DIM HS(V),SU(V),P(V),X(V),Y(V),Z(V)
80 PRINT: PRINT
90 FOR A=1 TO V
100 INPUT "pH VALUE    ";P(A)
110 NEXT A
120 CLS
130 VDU2
140 PRINT: PRINT: PRINT: PRINT N$: PRINT
150 PRINT "pH VALUE", TAB(15)"SO2",TAB(30)"HSO3",TAB(45)"SO3"
160 PRINT: PRINT
170 FOR A=1 TO V
180 HS(A)=10^(P(A)-1.77)
190 SU(A)=10^(P(A)-7.20)
200 SU(A)=SU(A)*HS(A)
210 T=1+SU(A)+HS(A)
220 X(A)=1/T*100
230 Y(A)=HS(A)/T*100
240 Z(A)=SU(A)/T*100
260 PRINT P(A),TAB(15);X(A),TAB(30);Y(A),TAB(45);Z(A)
270 NEXT A
280 VDU3
290 PRINT: PRINT
300 INPUT "(A)GAIN OR (E)ND    ";N$
310 IF N$="A" THEN CLEAR: GOTO 30
320 IF N$="E" THEN CLS: END
330 GOTO 300
```

APPENDIX 1: Computer program, written in B.A.S.I.C., used to calculate relative proportions of sulphur dioxide present in aqueous solution. The computer and printer used were, respectively, a B.B.C. Model B and an Epson RX 80.

```
3 REM * SP Maloney *
5 REM * Intracellular pH Via Sulphite Uptake *
10 MODE 0:@%=&00309
20 INPUT "YEAST I.D.    ";N$
30 INPUT "INTRACELLULAR VOLUME OF YEAST    ";IV
35 IV=IV*10^-6
40 VDU2
50 PRINT N$: PRINT: PRINT
60 PRINT "EXTERNAL TOTAL SO2", TAB(30)"SO2 UPTAKE",
TAB(60)"pH VALUE"
70 VDU3: CLS
80 INPUT "EXTERNAL TOTAL SO2    ";EC
90 IF EC=0 THEN GOTO 230
100 INPUT "UPTAKE MEASURED    ";UM
110 EC=EC*10^-6
120 UM=UM*10^-9
140 A=0.585*EC/100
150 A=A*IV/(1*10^-3-IV)
160 A=A*100/UM
170 A=100/A
180 A=LOG A+1.77
190 VDU2
200 PRINT EC,TAB(30);UM,TAB(60);A
210 VDU3
220 GOTO 80
230 INPUT "(A)GAIN OR (E)ND    ";N$
240 IF N$="A" THEN GOTO 20
250 IF N$="E" THEN CLS: END
260 GOTO 230
```

APPENDIX 2: Computer program, written in B.A.S.I.C., used to estimate intracellular pH values using sulphite-uptake and intracellular-volume data. The computer and printer used were, respectively, a B.B.C. Model B and an Epson RX 80.